Physicochemical and kinetic properties of human milk xanthine oxidoreductase

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Award date:
1998

Awarding institution:
University of Bath

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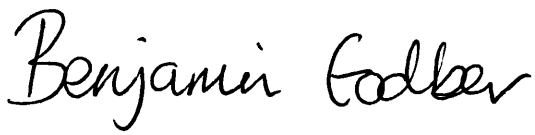
submitted by Benjamin L. J. Godber
for the degree of PhD
of the University of Bath
1998



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Dedicated to Rachel

Acknowledgements

I would like to thank my supervisors Dr Roger Harrison and Professor Robert Eisenthal for all their help during this project and for allowing me the opportunity to undertake this project. I would also like to thank the BBSRC for funding it. I am also extremely grateful for all the helpful discussions and invaluable practical support that Dr Mustapha Benboubetra, Professor Bob Bray, Dr Stephen Sanders, Dr Laurie Irons, Professor Garry Taylor, Dr Giuliano Siligardi and Patrick Turner have provided. For all their help and entertainment throughout my project I would also like to thank Richie, JJ, Arwen, Sue, Magali and Sharmy. Finally, I would like to thank all my family, especially Rachel, for their love and support .

Summary

An improved purification of xanthine oxidoreductase (XOR) is described, where yields of 20-30 mg of pure enzyme can be obtained per litre of frozen breast milk. The kinetic and physicochemical properties of purified human XOR were compared with those of the bovine milk enzyme, similarly prepared.

The low enzymic activity of human XOR towards xanthine and related substrates was confirmed and explained on the basis of a high proportion (>95%) of demolybdo enzyme. The deficit of molybdenum was associated with a lack of molybdenum cofactor and with a 30% loss of iron-sulphur centres. Of the low content of molybdenum-containing enzyme, approximately 60% was shown, on the basis of activity and resulphuration experiments, to consist of desulpho enzyme. The substrate specificity of human milk XOR was closer to that of human liver enzyme than to that of bovine milk XOR.

Human milk XOR showed several physicochemical differences from the bovine enzyme, notably a consistently higher apparent molecular weight, greater tendency to aggregate and higher affinity for heparin. Moreover the conversion of dehydrogenase to oxidase form was less complete than for bovine milk XOR.

The NADH oxidase activity of human milk XOR was investigated and a speculative recycling mechanism is proposed whereby, in the presence of NAD^+, the dehydrogenase form of XOR can generate significant amounts of superoxide and hydrogen peroxide in the course of oxidation of xanthine.

Abbreviations

AFR	activity to flavin ratio
AH	amino-hexyl
AOR	aldehyde oxidoreductase
APR	2-aminopurine ribonucleoside
ATP	adenosine triphosphate
Bicine	N,N-bis(2-hydroxyethyl)glycine
BMXOR	bovine milk xanthine oxidoreductase
BSA	bovine serum albumen
CD	circular dichroism
CHES	2(N-cyclohexylamino)ethanesulphonic acid
DH	dehydrogenase
DLS	dynamic light scattering
D_T	translational diffusion coefficient
DTDP	4,4'-dithiodipyridine
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbant assay
EPR	electron paramagnetic resonance
FAD	flavin adenine dinucleotide
$FADH^{\bullet}$	FAD neutral semiquinone
$FADH_2$	reduced FAD
Fe-S	iron-sulphur centre
FPLC	fast protein liquid chromatography
HLXOR	human liver xanthine oxidoreductase
HMXOR	human milk xanthine oxidoreductase
HPLC	high pressure liquid chromatography
I-R	ischemia-reperfusion
ICAM	intracellular adhesion molecule
ICP	inductively coupled plasma
IFN	interferon
IL	interleukin

kDa	kilodalton
K_R	retardation coefficient
MALDI	matrix assisted laser desorption ionisation
MES	2-(N-morpholino)ethanesulphonic acid
MFGM	milk fat globule membrane
Moco	molybdenum cofactor
MOPS	3-(n-morpholino)propanesulphonic acid
MS	mass spectrometry
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced NAD^+
NBT	nitroblue tetrazolium chloride
O	oxidase
PAGE	polyacrylamide gel electrophoresis
PES	phenazine ethosulphate
PFR	protein to flavin ratio
PMSF	phenylmethylsulphonyl fluoride
Rf	relative mobility
R_H	hydrodynamic radius
RNA	ribose nucleic acid
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
TCA	trichloroacetic acid
TEMED	N, N, N', N',-tetramethylethylenediamine
TNF	tumour necrosis factor
TOF	time of flight
UV	ultraviolet
XDH	xanthine dehydrogenase
XO	xanthine oxidase
XOR	xanthine oxidoreductase

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2 Aims

Although mammalian XOR from bovine milk and rat liver has been well characterised, relatively little is known about the human enzyme. The aims of the present work were to develop an improved purification procedure from human milk and to compare the kinetic and physicochemical properties of the purified human enzyme with those of the enzyme from bovine milk. By these means, it was hoped to clarify the molecular bases and physiological relevance of differences between the enzyme from human milk and those from other mammalian species.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

Bovine Milk Xanthine Oxidoreductase was obtained from Biozyme, Blaenavon, Gwent. "Protogel" (Acrylamide-bisacrylamide stock solution) was obtained from National Diagnostics, Atlanta, Georgia, USA. Bio-rad protein assay reagent concentrate was obtained from Bio-rad, Hemel Hempstead, Hertfordshire. Dimercaptotoluene was obtained from Fluka, Gillingham, Dorset. All other chemicals were obtained from Sigma-Aldrich, Poole, Dorset; BDH, Poole, Dorset; and Fisher Scientific, Loughborough, Leicestershire.

3.1.2 Column chromatography

AH Sepharose, Mono Q Hi-trap columns, Hi Load 16/60 Prep Grade Superdex 200 gel filtration column and Sephadex G25 PD10 buffer exchange columns were obtained from Pharmacia, Uppsala, Sweden. Heparin-agarose (Type I) was obtained from Sigma, Poole, Dorset. Syringe Filters were obtained from Whatman, Maidstone, Kent.

3.1.3 Equipment

PAGE was carried out using either the Protean II or Mini-Protean II electrophoresis tanks and plates, obtained from Biorad, Hemel Hempstead, Hertfordshire. Column chromatography was performed using a Pharmacia FPLC system from Pharmacia, Uppsala, Sweden. HPLC was carried out on a Waters HPLC system equipped with a Waters multiwavelength detector from Waters chromatography division, Millipore corporation, Massachusetts.

3.1.4 Instruments

Spectrophotometric assays were performed using either a Cecil CE 6600 Multimode Computing UV Spectrophotometer or a Cecil CE 272 linear readout UV Spectrophotometer. Spectra were taken using a Cecil CE 6600 Multimode Computing UV Spectrophotometer. Fluorimetric assays were done using a Perkin-Elmer LS-5B Luminescence Spectrometer. Dynamic light scattering studies were performed using an Oros Instruments M801 Molecular Size Detector, Oros instruments, Slough, Berkshire.

3.2 General Methods

3.2.1 Dye binding protein estimation

Protein estimations were done according to the method of Bradford (1976). Commercially-available BMXOR or BSA were used as the standard for estimations of protein in solutions of pure enzyme or crude extracts respectively. The standard, diluted to a range of concentrations from 0 to 100 μg/ml, and unknown samples at appropriate dilutions (100 μl) were added to BIO-RAD protein assay reagent (0.9 ml) in a 1 ml plastic cuvette, mixed by inversion and left for 10 min to allow colour development. The absorbance at 595 nm was determined and protein concentration was calculated from a linear standard curve.

3.2.2 Calculation of XOR concentration from spectra

XOR concentration was calculated from the A_{450} using extinction coefficients estimated in Section 7.3.1. If no extinction coefficient had been estimated, an ϵ of 36000 M^{-1} cm^{-1} was used, which assumes fully functional enzyme (Bray, 1975).

3.2.3 Xanthine oxidase activity assay

The total xanthine oxidase activity was determined by measuring the rate of oxidation of xanthine to uric acid spectrophotometrically at 295 nm, using an absorption coefficient of 9600 M^{-1} cm^{-1} (Avis *et al.*, 1956 a). Assays were performed in acrylic cuvettes (1 ml) at 25°C in air-saturated 50 mM Na-Bicine, pH 8.3, unless otherwise stated, containing 100μM xanthine and 500μM NAD^+.

3.2.4 Oxidase content

The oxidase content was determined by measuring the rate of oxidation of xanthine to uric acid spectrophotometrically, as described in Section 3.2.3, at 25°C in air-saturated 50 mM Na-Bicine, pH 8.3, containing 100μM xanthine in the absence of 500μM NAD^+. Percentage oxidase was calculated by dividing the rate in the absence of NAD^+ which was assumed to be the oxidase, by that in the presence of NAD^+, which was assumed to be the sum of the oxidase and dehydrogenase. This fraction was expressed as a percentage.

3.2.5 NADH oxidase assays

NADH oxidase activity was determined by measuring the rate of oxidation of NADH to NAD^+ spectrophotometrically at 340 nm, using an absorption coefficient of 6220 M^{-1} cm^{-1} for NADH (Horecker & Kornberg, 1948). Assays were performed in acrylic cuvettes at 25°C in air-saturated 50 mM Na-Bicine, pH 8.3, unless otherwise stated, containing 100 μM NADH.

3.2.6 Fluorescence assay for xanthine oxidase activity

The more sensitive fluorescence assay of Beckman *et al.* (1989) was used when there was too little activity to be measured using the standard spectroscopic assay or when turbidity of the solution made the spectroscopic assay unfeasible. The assay utilises the conversion of pterin by XOR to the fluorescent product, isoxanthopterin. Pterin is oxidised to isoxanthopterin at the molybdenum site by the same mechanism that oxidises xanthine to urate.

Assays were carried out in a Perkin-Elmer LS-5B Luminescence Spectrometer at room temperature, with an excitation wavelength of 345 nm and emission wavelength of 390 nm. Sample (10 μl), was added to 50 mM potassium phosphate, pH 7.4, (970 μl) in 1 ml quartz fluorimeter cells and fluorescence monitored to ensure a stable baseline. Pterin

(1 mM stock solution, 10 μl) was added, mixed by inversion and fluorescence monitored to give the oxidase activity. Methylene blue (1 mM stock solution, 10 μl) was added, mixed by inversion and fluorescence monitored to give the total activity (XO+XDH). Allopurinol (4 mM stock solution, 10μl) was added, mixed by inversion and fluorescence was monitored to confirm that the production of fluorescent product was allopurinol-inhibitible. This also allows calibration by adding known amounts of isoxanthopterin, which act as an internal standard and takes into account the differences observed in fluorescence quenching and light scatter.

3.2.7 Calculation of AFR

AFR [Activity (units/l) / A_{450}] is a convenient method to describe the functionality of an XOR sample (Avis *et al.*, 1955). AFR is calculated by dividing the rate of increase of A_{295} (AU/min) of the assay described in Section 3.2.3 by the A_{450} of sample as diluted in the assay. An AFR value of 210 has become accepted to indicative of 100% functional enzyme (Massey *et al.*, 1970).

3.2.8 Calculation of PFR

PFR (A_{280} / A_{450}) is a convenient method to describe the purity of an XOR sample (Avis *et al.*, 1955). PFR is calculated by dividing A_{280} by the A_{450} of the sample. A PFR value of 5 has become accepted as representative of highly pure XOR.

4 Purification of Human and Bovine Milk Xanthine Oxidase

4.1 Introduction

The purification of xanthine oxidoreductase (XOR) from bovine milk was first reported in 1924 by Dixon & Thurlow, and in 1939, separately Ball and Corran *et al.* obtained enzyme of sufficient purity to show the presence of flavin and the possibility of another chromophore in the visible spectrum. By 1955, Avis *et al.* had developed a purification procedure which yielded crystalline XOR with a protein (280 nm) to flavin (450 nm) ratio (PFR) of 5.0-5.2, which later became accepted as indicative of a very high level of purity. Subsequently, XOR has been purified from a wide variety of species and sources. The early purification procedures were relatively harsh by current standards, involving solvent extraction, drying, acid and heat treatment and exposure to proteases (Massey & Harris, 1997).

Bovine milk has most commonly been used as a source of XOR as it contains relatively high amounts of the enzyme, with fewer contaminating proteins than do other tissue sources. The procedure used by Avis *et al.* (1955), yielding XOR of high purity, required the proteolysis, using pancreatin, of buttermilk. Proteolysis was found to selectively denature

and precipitate contaminating casein and has also been implicated in the release the enzyme from the milk fat globule membrane (Briley & Eisenthal, 1974). The widespread use of proteases delayed the identification of the dehydrogenase form of XOR (XDH) until 1969 when Stirpe & Della Corte described an apparent activation of rat liver XOR in the presence of proteases and established the phenomenon of protease-induced irreversible XDH to XO conversion (See General Introduction).

Many different chromatographic methods have been employed in the purification of XOR. Calcium phosphate chromatography generally gives a large increase in purity with little loss of total activity (Avis *et al.*, 1955) and has been used successfully in preparations of XOR from a wide variety of sources. However, in the experience of our laboratory, inconsistency in binding capacity between batches and short effective life make this material unreliable. An important advance involved the use of folate as an affinity ligand (Nishino *et al.*, 1981). Folate, which can be covalently bound to Sepharose, is a competitive inhibitor of XO with a K_i of 42 μM (Nishino & Tsushima, 1986), convenient for use as an affinity ligand. Folate has the particular advantage that it only binds to molybdo (molybdenum-containing) enzyme so facilitating the fractionation of active and inactive forms which occur in most preparations (See General Introduction and Chapter 10).

There are few reports of the purification of XOR from human sources. Human milk was long thought to contain very little XOR, because of the low enzymic activity towards xanthine in this tissue. Zikakis *et al.* (1983) described a preparation of highly impure enzyme, obtained by a combination of mild non-ionic detergent and multiple chromatographic techniques. Six years later, Graham *et al.* (1989) briefly reported a purer product using salt precipitation followed by hydroxylapatite chromatography. Krenitsky *et al.* (1986) reported purification of a highly proteolysed enzyme from post-mortem human liver using batchwise calcium phosphate chromatography followed by the use of a novel affinity ligand, 9-(p-amino-ethoxyphenyl) guanine coupled to Sepharose 4B.

Abadeh *et al.* in 1992 described a preparation from human milk of relatively pure XOR. Their procedure was based on that of Nakamura & Yamazaki (1982) for bovine milk and involved the use of a high ionic strength buffer to liberate XOR from the cream and ammonium sulphate precipitation followed by calcium phosphate chromatography. This procedure has subsequently been modified in our laboratory, most recently resulting in that described by Sanders *et al.* (1997). In this preparative method, calcium phosphate chromatography is replaced by heparin chromatography; an advance based on findings of Adachi *et al.* (1993) who in the course of studies on the binding of XOR to cell surfaces, demonstrated binding of HMXOR to heparin. Although the

application of heparin binding in purification procedures was not suggested by these workers, the potential was clearly evident. XOR binds to heparin, a sulphated glycosaminoglycan, via lysine and arginine rich peptides (Fukushima *et al.*,1995).

4.2 Materials and Methods

4.2.1 Materials

Frozen human milk was kindly donated by mothers in the Special Care Baby Units of the Royal United Hospitals, Bath, Southmead Hospital, Bristol; Bristol Royal Infirmary and Princess Margaret Hospital, Swindon. Fresh human milk was kindly provided by mothers in the Bath and Chippenham Area. Fresh bovine milk was obtained from a local dairy herd in Claverton, Bath.

4.2.2 Purification of BMXOR and HMXOR according to Sanders *et al.* (1997).

To fresh, or thawed frozen milk, 1 mM EDTA and 0.1 mM phenylmethyl-sulphonyl fluoride (PMSF) were added, and the mixture was stirred for 20 min before centrifuging (3000g, 30 min) at 4°C. All subsequent operations were carried out at 4°C. The cream was resuspended (50% v/v) in 0.2 M

K_2HPO_4 containing 1 mM EDTA and 0.1 mM PMSF, with stirring for 20 min. The suspension was centrifuged (3000g, 30 min) and the aqueous supernatant was collected, filtered through glass wool and mixed with 15% (v/v) butan-1-ol. 15% (w/v) Ammonium sulphate was added slowly and left stirring for 1 h before centrifuging (3000g, 20 min) and filtering the subnatant through glass wool. A further 20% (w/v) ammonium sulphate was added and allowed to stir for 45 min before centrifuging (3000g, 30 min). The precipitate was resuspended in heparin chromatography buffer (H-buffer) (20 ml) (25 mM sodium-phosphate, pH 7.4, for HMXO and 25 mM MES, pH 6.0, for BMXO containing 1 mM EDTA and 0.1 mM PMSF) and dialysed against the same buffer overnight. Any remaining precipitate was removed by centrifugation (27000g, 1 h) followed by filtration through a 0.22 μm filter, to yield a crude enzyme preparation.

A heparin column was prepared by packing heparin immobilised on cyanogen bromide-activated 4% cross-linked agarose in H-buffer into a column (1 cm × 6 cm) under gravity. The column was washed with H-buffer containing 1 M NaCl and equilibrated with H-buffer (20 ml) prior to use. The crude enzyme preparation, in H-buffer, was applied to the column at a flow rate of 20 ml/h. The column was washed with H-buffer (20 ml), containing 0.1 M NaCl and the enzyme was then eluted in H-buffer containing 0.4 M NaCl for HMXOR and 0.25 M NaCl for BMXOR.

Further purification could be effected by using Mono Q anion exchange chromatography. XOR in 50 mM Na-Bicine buffer, pH 8.3, containing 50 mM NaCl was filtered through a 0.22 μm syringe filter and applied to an equilibrated 1ml Hi-Trap Mono Q ion exchange column (Pharmacia) at a flow rate of 1 ml/min. The column was eluted by a gradient of increasing salt concentration from 0.05 M to 0.5 M NaCl in 50 mM Na-Bicine buffer, pH 8.3, over 30 min. Fractions were collected and those containing the enzyme were pooled.

4.2.3 Folate Affinity Chromatography

Folate column preparation is that of Nishino *et al.* (1981) with slight modification. To swollen and washed (0.5M NaCl) AH Sepharose 4B (Pharmacia) (4 g dry weight) 2 mM folic acid in 50% dimethylformamide, pH 5.8, (30 ml) and 1-ethyl-3-(3-dimethylpropyl) carbodi-imide (200 mg) was added and the slurry was gently agitated at room temperature for 3 h or until the supernatant was not visibly yellow. This coupling procedure was repeated 3 times until the supernatant remained yellow. The gel was then washed sequentially with 50% DMF, 10 mM NaOH and 0.1M pyrophosphate buffer pH 8.5. The gel was packed into a column (1 cm × 6 cm) which was covered with aluminium foil to exclude light, washed and stored at 4°C in 0.1M pyrophosphate buffer, pH 8.5, until use.

The folate Sepharose column was equilibrated in Buffer A [20% (v/v) 0.1 M sodium pyrophosphate, pH 8.5, containing 0.2 mM EDTA and 80% (v/v) 50 mM Tris-HCl, pH 7.8, containing 0.2 mM EDTA] before use. BMXOR in Buffer A was applied to the column at a flow rate of 0.5 ml/min until the column was visibly brown approximately one third of the way down. The column was then washed in Buffer A followed by Buffer B [30% (v/v) 0.1 M sodium pyrophosphate, pH 8.5, containing 0.2 mM EDTA and 70% (v/v) 50 mM Tris-HCl, pH 7.8, containing 0.2 mM EDTA] until the UV absorbance had returned to baseline, at which time Buffer B, containing 100 mM hypoxanthine was used to elute molybdo XOR.

4.3 Results

4.3.1 Xanthine Oxidase Purification

HMXOR was initially purified from frozen human breast milk (1 l) using the procedure of Sanders *et al.* (1997), as described in Section 4.2.1, up to and including heparin chromatography. Using this procedure 5 mg HMXOR was obtained with PFR 5.1 - 5.5 and containing approximately 70% oxidase activity (Table 4.1). A typical elution profile of HMXOR from heparin-agarose is shown in Fig. 4.1.

Fraction	Volume (ml)	Total Activity (nmol min^{-1})	Total Protein (mg)	Specific Activity (pmol min^{-1} mg^{-1})	Yield (%)
Milk	1000	1180	17880	66	100
Resuspended Cream	267	611	2008	305	52
K_2HPO_4 wash	240	444	432	1028	38
1st $(NH_4)_2SO_4$ subnatant	240	398	192	2075	34
Purified XOR	3	120	5	24784	10

Table 4.1. Purification of HMXOR using the procedure of Sanders *et al.* (1997) (Section 4.2.2). Protein concentrations and pterin oxidase activity were determined as described in Sections 3.2.1 and 3.2.6 respectively.

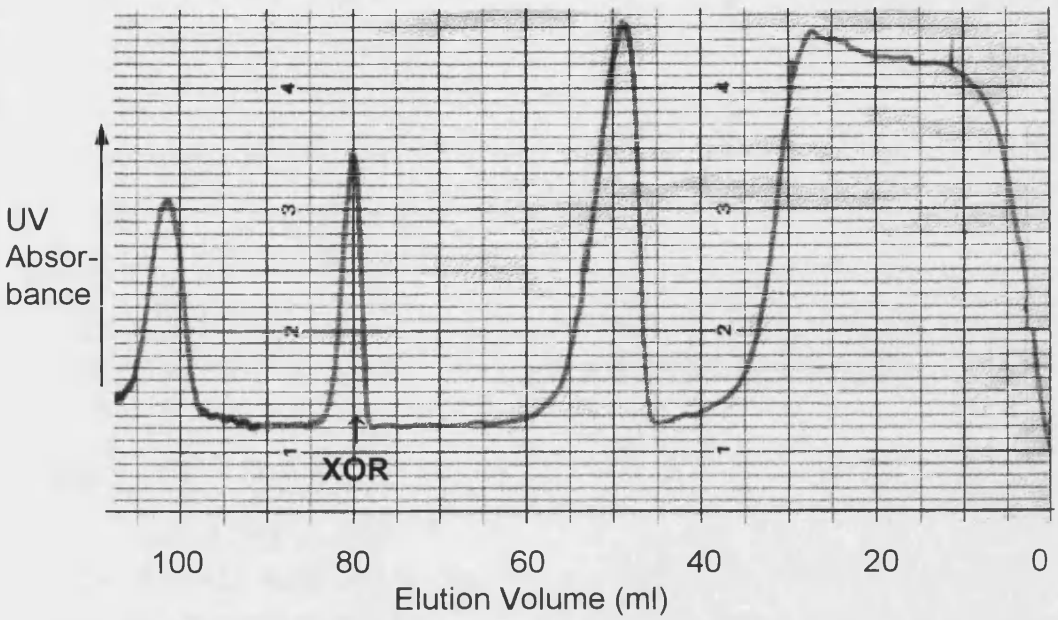


Figure 4.1. Elution profile of crude HMXOR from a heparin agarose column. The column eluate was monitored using a UV detector, linked to a chart recorder.

4.3.2 Effect of DTT on the Purification of HMXOR

The effects of DTT on the early stages of purification of HMXOR were investigated by following the sequence of operations outlined in Fig. 4.2. Enzymic activity of Fractions 2 to 6 are shown in parentheses as percentages of the total starting activity shown as 100% in Fraction 1. Comparison of Fraction 2 vs. Fraction 3, also of Fraction 5 vs. Fraction 6, shows clearly that addition of DTT leads to greatly increased release of XOR from the cream. A second very important point emerges from this experiment. This is that initial stirring of thawed milk, prior to centrifugation, leads to extensive loss of XOR into the subnatant (Fraction

3). It was, accordingly, decided to modify the preparation of Sanders *et al.* by centrifuging milk immediately after thawing (avoiding stirring) and to include DTT in the subsequent stirred washing of separated cream.

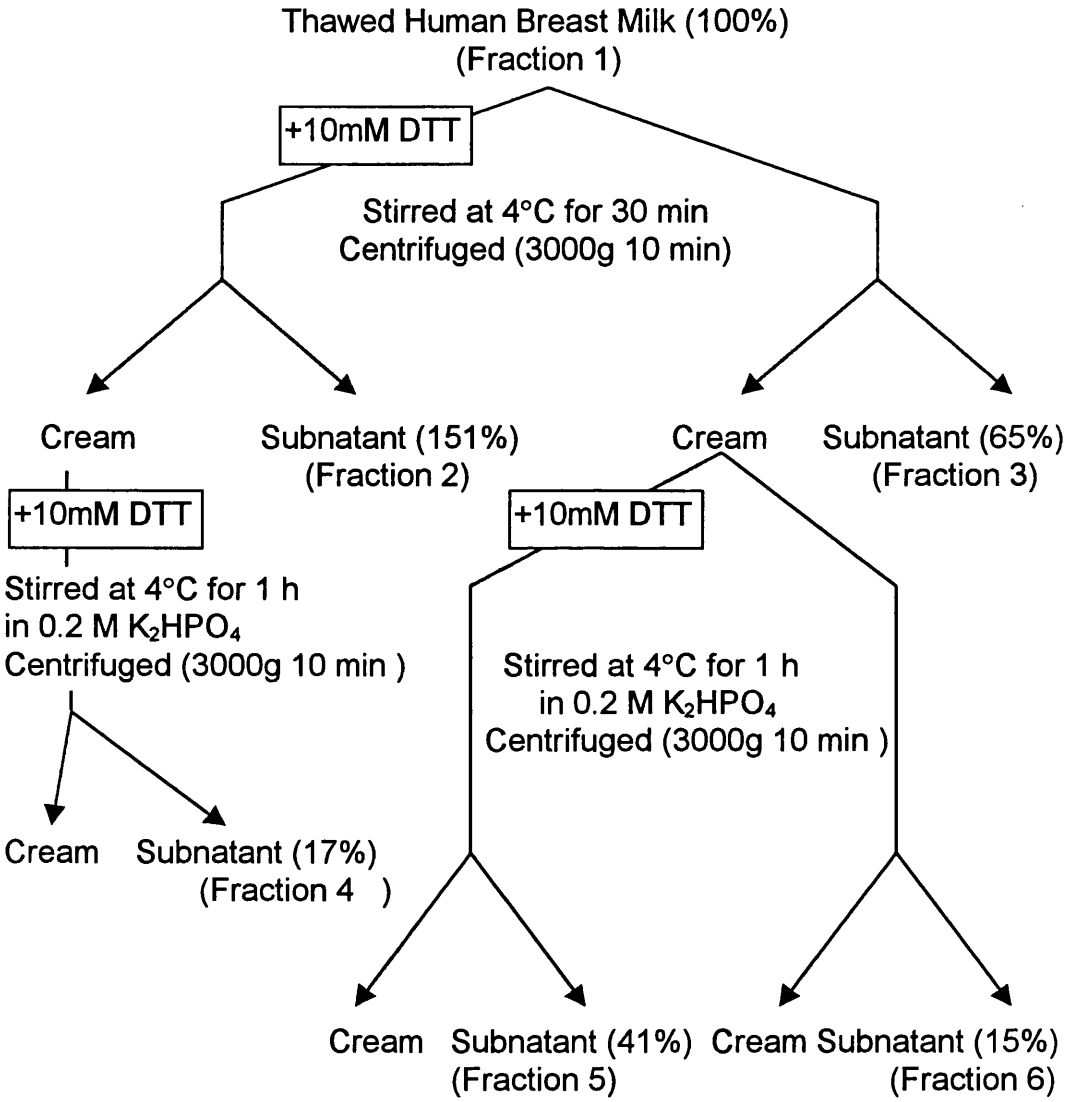


Figure 4.2. Schematic diagram of the procedures used to monitor the effect of DTT on the purification of HMXOR. Pterin oxidase activity was determined as described in Section 3.2.6 and expressed as percentages of the total starting activity of Fraction 1.

4.3.3 Improved Xanthine Oxidase Purification

In view of the results outlined in Section 4.3.2, the preparation of Sanders *et al.* (1997), was modified. Milk was centrifuged immediately after thawing and 10 mM DTT was added to the buffer used to resuspend the cream and maintained in all subsequent operations. Table 4.2 shows the purification details for HMXOR. It is noteworthy that the total enzyme activity of HMXOR increases following initial DTT treatment; an observation that will be discussed later. The improved purification procedure was also applied to BMXOR, as shown in Table 4.3.

Fraction	Volume (ml)	Total Activity (nmol min^{-1})	Total Protein (mg)	Specific Activity (pmol min^{-1} mg^{-1})	Yield (%)
Milk	1000	1230	17400	71	100
Resuspended Cream	288	1281	1381	927	104
K_2HPO_4 wash	213	1661	618	2690	135
1st $(NH_4)_2SO_4$ subnatant	237	1235	190	6500	100
Purified XOR	5	554	21	26829	45

Table 4.2. Purification of HMXOR using the modified procedure (Section 4.3.3). Protein content and enzyme activity were determined as described in the legend to Table 4.1.

Fraction	Volume (ml)	Total Activity (nmol min^{-1})	Total Protein (mg)	Specific Activity (pmol min^{-1} mg^{-1})	Yield (%)
Milk	1000	27800	26400	1053	100
Resuspended Cream	235	20728	1009	2535	75
K_2HPO_4 wash	162	13492	518	26031	49
1st $(NH_4)_2SO_4$ subnatant	164	11502	279	41176	41
Purified XOR	4	4463	24	186417	16

Table 4.3. Purification of BMXOR using the modified procedure (Section 4.3.3). Protein content and enzyme activity were determined as described in the legend to Table 4.1.

4.3.4 Further Purification Using Mono Q Ion Exchange

Preparations of XOR after heparin chromatography often showed a contaminating band at 70 kDa on SDS-PAGE and a PFR above 5. Further purification by anion exchange chromatography used by Sanders *et al.* was re-examined. Preliminary experiments established that there was little difference between the binding and elution of HMXO over a range of pH from 8 to 9. Na-Bicine, pH 8.3, containing 0.05 M NaCl was chosen for Mono Q anion chromatography with a linear elution gradient of NaCl from 0.05 M to 0.5 M, with a step at 0.2 M NaCl, in Na-Bicine, pH 8.3, over 30 ml. Figure 4.3 shows a typical elution profile of HMXOR from Mono Q.

After Mono Q chromatography HMXOR typically had a PFR of 5 to 5.1. If it is assumed that pure enzyme has a PFR of 5 (Bray, 1975), this corresponds to a purity of 98% to 100%, which is further validated by the enzyme running as a single band on SDS PAGE.

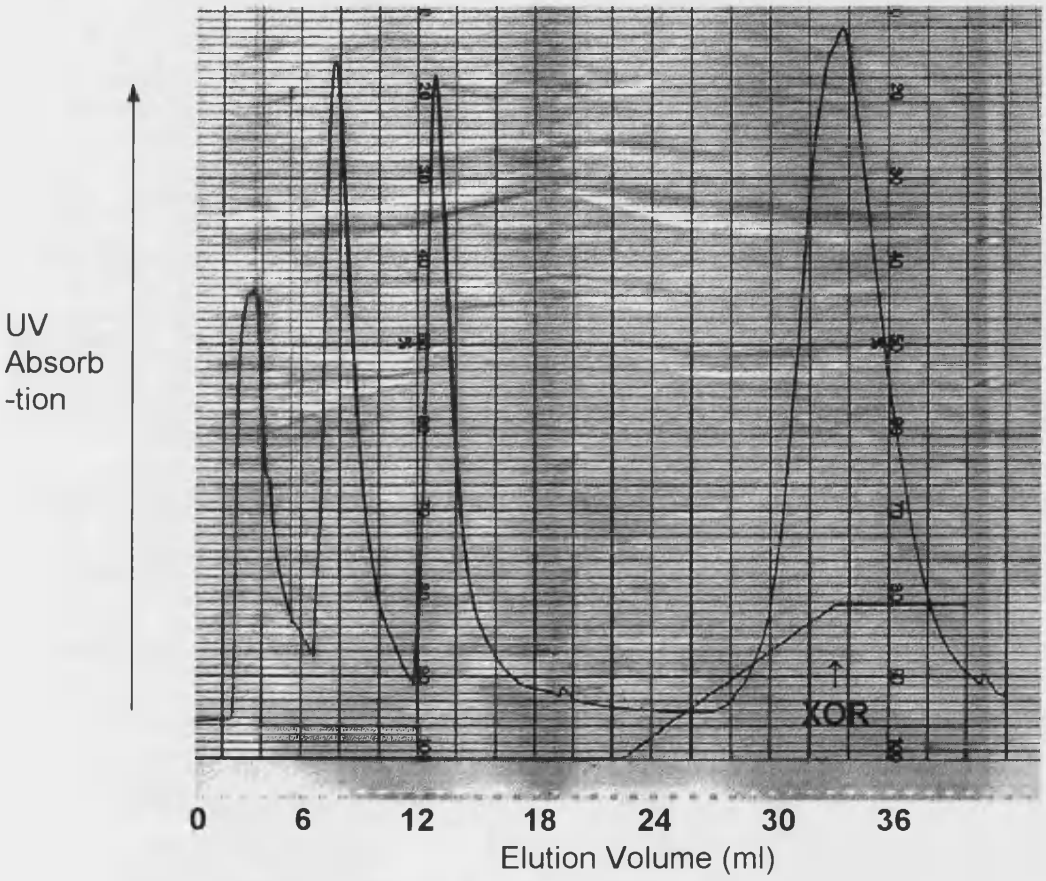


Figure 4.3 Elution profile of HMXOR from a Hi-Trap Mono-Q column. The column eluate was monitored using a UV detector, linked to a chart recorder. The NaCl gradient and step at 0.2M is shown by the line starting at 22ml.

4.3.5 Folate Chromatography

Folate chromatography was carried out to enrich BMXOR in molybdo enzyme and to provide some samples of low molybdenum content for comparative study with HMXOR. On all samples studied, two successive chromatography steps were carried out. The first affinity chromatography step (Figure 4.4) shows that unbound enzyme has a low AFR, but, as the column is washed, the AFR progressively increases. Upon elution with hypoxanthine, most of the XOR active fraction elutes. However the last few fractions were found to have a very low AFR which may reflect the

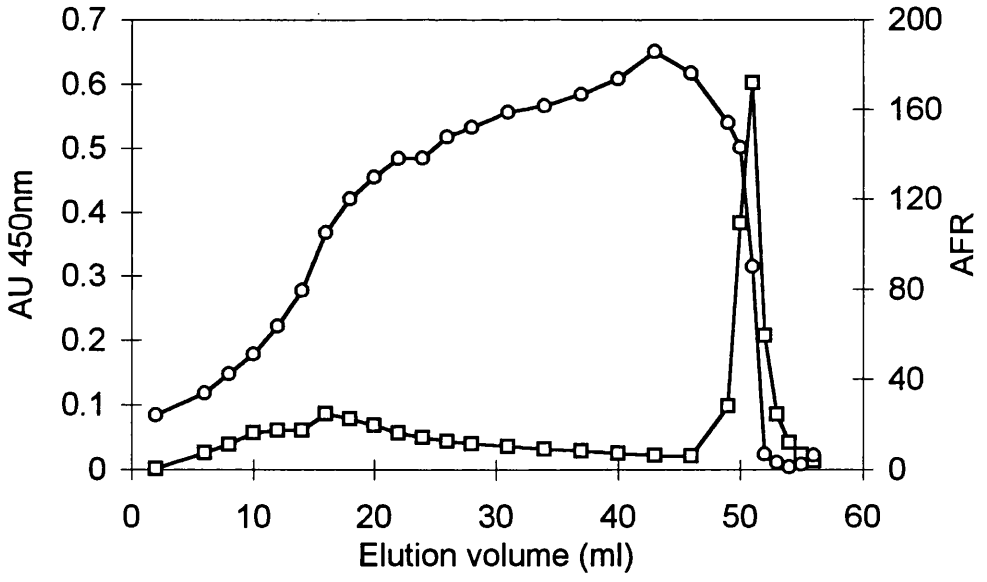


Figure 4.4. Elution Profile of BMXOR from a folate Sepharose column. Fractions were collected, and for each, the absorbance at 450nm was measured (□) and the AFR (O) was determined as described in Section 3.2.7.

fact that desulpho XOR, which binds folate may have either a lower affinity for hypoxanthine or a higher affinity for folate than active XOR.

The second folate affinity chromatography step (Figure 4.5) shows that enzyme that doesn't bind has a very low AFR and during the hypoxanthine elution the AFR increases, approaching that for 100% functional enzyme of 210 (Massey *et al.*, 1970; Edmonson *et al.*, 1972).

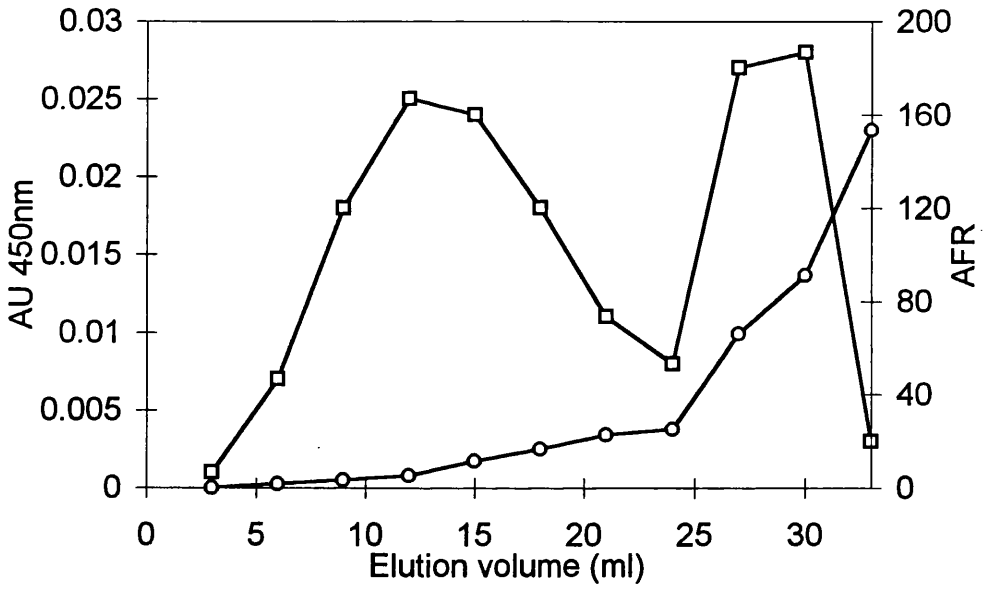


Figure 4.5. Elution Profile of demolybdo-enriched BMXOR from a folate Sepharose column. Fractions were collected, and for each, the absorbance at 450nm was measured (□) and the AFR (O) was determined as described in Section 3.2.7.

The specific activities of the enriched molybdo and demolybdo BMXOR fractions for xanthine and NADH oxidation were assayed (Table 4.4), demonstrating dramatic differences in the molybdenum-dependant xanthine activity and similar NADH oxidase activities.

	Xanthine Oxidase Activity (nmol min^{-1} mg^{-1})	NADH Oxidase Activity (nmol min^{-1} mg^{-1})
Molybdo BMXOR	1538 ± 0	48 ± 1
Demolybdo BMXOR	61 ± 2	45 ± 2

Table 4.4 Xanthine and NADH oxidase activities of molybdo- and demolybdo-BMXOR. Enzyme concentration, xanthine oxidase activity and NADH oxidase activity were determined in air-saturated 50 mM Na-Bicine, pH 8.3, as described in Sections 3.2.2, 3.2.3 and 3.2.5 respectively.

4.4 Discussion

Using the procedure of Sanders *et al.* (1997), frozen human milk only yielded approximately 5 mg XOR protein per l milk. In attempts to prepare XOR in the dehydrogenase form (XDH), DTT was included in all buffers and, although overall yields remained similar to those obtained in the absence of DTT, it was observed that DTT facilitated the release of enzyme from the cream. This effect was confirmed in experiments outlined in Fig. 4.2. These experiments also highlighted the fact that initial stirring of thawed milk prior to the first centrifugation step led to considerable loss of enzyme activity from the cream. In the light of the above findings, the preparation of Sanders *et al.* was modified in two major ways. Firstly, frozen human milk was thawed and immediately centrifuged to obtain cream without previous stirring. Secondly, DTT was included in the subsequent washing step to maximise release of enzyme from the cream. As a consequence, HMXOR of undiminished purity was obtained at approximately four times the previous yield.

It is of interest that, in previous years, the preparation of Sanders *et al.* has always given much higher yields of BMXOR than of HMXOR. In retrospect, this may well have been a consequence of the use of fresh bovine milk, in contrast to frozen human milk routinely employed. The logistics of human milk collection largely dictate the use of frozen milk,

which after thawing, is likely to have suffered disruption of the membrane surrounding the fat globules and to contain HMXOR largely in the oxidase form. XOR is associated with this membrane and may be released more readily from disrupted than from intact membrane, *i.e.* from thawed human milk than from intact membranes of fresh bovine milk. As a consequence, enzyme was probably largely lost in the initial stirring step in the case of human milk. In confirmation of this idea, the preparation of Sanders *et al.* has always given much higher yields when fresh human milk was used.

The nature of the association of XOR with the milk fat globule membrane is unclear. Milk fat globules are, however, known to be surrounded by glycosylaminoglycans (Welsch *et al.*, 1988; Buchheim *et al.*, 1988) to which XOR binds (Adachi *et al.*, 1993; Radi *et al.*, 1997). Elsewhere in this thesis, it is shown that HMXO binds more tightly to heparin than does HMXDH (Chapter 8) and it is tempting to explain the facilitated release of XOR from the milk fat globule effected by DTT in terms of conversion of XO to XDH and consequently decreased affinity to glycosaminoglycans.

A further point worthy of note is the increase in overall XOR activity observed following release from the cream. Two factors may contribute to this. Firstly, membrane association of XOR has been shown (Briley & Eisenthal, 1974; Briley & Eisenthal, 1975) to decrease activity to xanthine (and presumably pterin). Secondly, DTT in the releasing buffer converts

the predominantly oxidase form of HMXOR to the dehydrogenase form, which, as shown in Section 8.2, shows intrinsically higher specific activity.

5 Molecular Weight Estimation

5.1 Introduction

It has been previously reported that HMXOR has lower mobility than BMXOR on SDS-PAGE (Abadeh *et al.*, 1992). In this chapter I describe the estimation of the molecular weights for HMXOR and BMXOR using a variety of well-established methods. These methods, with the exception of SDS-PAGE, estimate the molecular weight using the Stoke's radius, and assume a roughly spherical conformation.

5.2 Materials and methods

5.2.1 SDS-PAGE

SDS-PAGE was carried out according to the method of Laemmli (1970). 10% and 7.5% Resolving gels consisted of Protogel [stock solution of 25% (w/v) acrylamide:bisacrylamide (40:1)] 40% (v/v) or 30% (v/v) respectively, in 0.375 M Tris-HCl, pH 8.8, containing 0.1% (w/v) SDS, polymerised by the addition of TEMED (0.4 µl/ml) and 0.1% (w/v) ammonium persulphate. 5% stacking gel consisted of Protogel [stock solution of 25% (w/v) acrylamide:bisacrylamide (40:1)] 20% (w/v) in 0.125

Tris-HCl, pH 6.8, containing 0.1% (w/v) SDS, polymerised by the addition of TEMED (0.4μl/ml) and 0.1% (w/v) ammonium persulphate. Reservoir buffer was 25 mM Tris-HCl, pH 8.3, containing 0.192 M glycine and 0.1% (w/v) SDS. Samples, in low ionic strength buffer, were mixed 1:1 with sample buffer [62.5 mM Tris-HCl, pH 6.8, containing 0.1% (w/v) SDS, 50% (v/v) glycerol, 0.02% (w/v) bromophenol blue and mercaptoethanol (10 μl/ml)] and denatured in a boiling water bath for 1.5 min before loading onto the gel. Gels were run at a constant voltage of 200 volts until the dye front reached the bottom of the gel. The stacking gel was removed and the resolving gel stained with staining buffer [distilled water containing 35% (v/v) methanol, 10% (v/v) acetic acid and 0.1% (w/v) coomassie Brilliant Blue dye] for 1 h, then de-stained using de-staining buffer [distilled water containing 5% (v/v) methanol and 10% (v/v) acetic acid].

Molecular weights were estimated by construction of a standard curve of molecular weight against the log R_f (relative mobility, calculated from the migration distance of the protein divided by that of the dye front) of the high molecular weight standard mixture (Sigma).

5.2.2 Native-PAGE

5%, 6%, 7% and 8% Gels and buffers were prepared as described in Section 5.2.2 with the omission of SDS and the concentration of acrylamide:bisacylamide was varied accordingly. Samples, in 25 mM sodium phosphate, pH 7.4, were mixed 1:1 with sample buffer and loaded onto the gels. Gels were electrophoresed at 5 milliamps until the marker dye was seen to have passed through the stacking gel and then at 10 milliamps until the marker dye had reached the bottom of the gel. Gels were stained for protein as described in Section 5.2.2.

Non-denaturing protein molecular weight marker kit (sigma), was run on the same gel as the sample for molecular weight determinations. The R_f was calculated (Section 5.2) and the K_R calculated from the slope produced by plotting $100 \times \text{Log} (R_f \times 100)$ against the % gel concentration for each protein. The molecular weights were estimated by constructing a standard curve of log $-K_R$ plotted against Log molecular weight.

5.2.3 Gel filtration

Sample (1 ml) was loaded onto a Hi Load 16/60 Prep Grade Superdex 200 gel filtration column, capable of separating molecular weights of 10 - 600 kDa, and chromatographed at a rate of 1 ml/min. Standards, dextran blue, yeast alcohol dehydrogenase, bovine serum albumin, carbonic

anhydrase and dinitrophenyl lysine, were used to calibrate the column for molecular weight determination. Molecular weights were estimated by constructing a standard curve of elution volume against log molecular weight.

5.3 Results

5.3.1 SDS-PAGE

HMXOR and BMXOR, the latter both prepared by myself and purchased from Biozyme laboratories were run on SDS-PAGE (Section 5.2.1). Fig. 5.1 shows a stained 7.5% gel from which it can be clearly seen that HMXOR runs with a lower mobility than does BMXOR. Log R_f values were plotted (Fig. 5.2), and molecular weights of HMXOR and BMXOR estimated (Table 5.1).

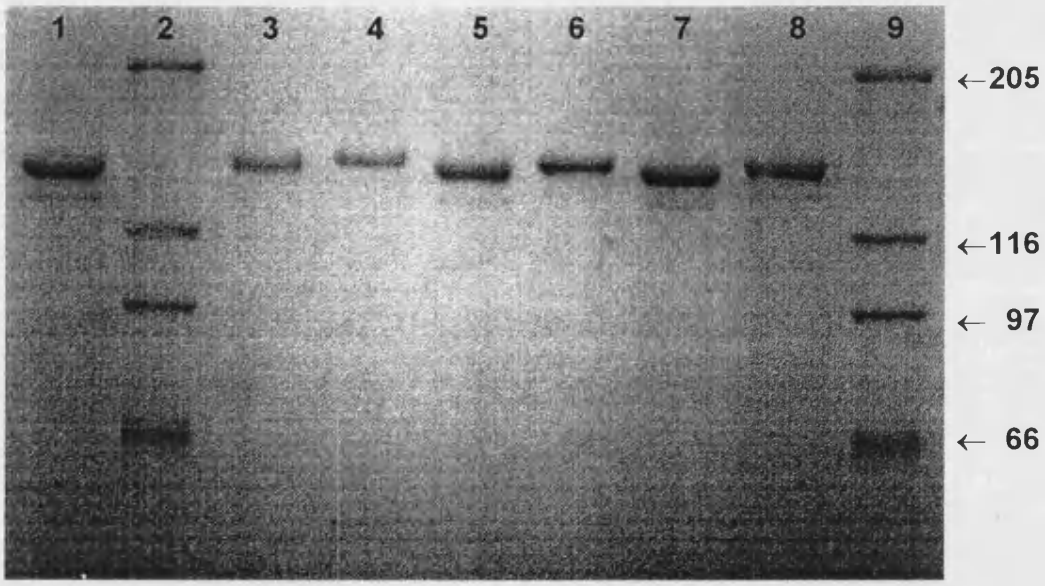


Fig. 5.1. SDS-PAGE of HMXOR and BMXOR. Different amounts of HMXOR and BMXOR were run on a 7.5% SDS-PAGE gel and stained for protein as described in Section 5.2.1. Lanes 1, 3, 5 and 7, BMXOR 15 μg, 5 μg, 10 μg, 15 μg, lanes 4, 6, and 8, HMXOR 5 μg, 10 μg, 15 μg, lanes 2 and 9, High molecular weight markers.

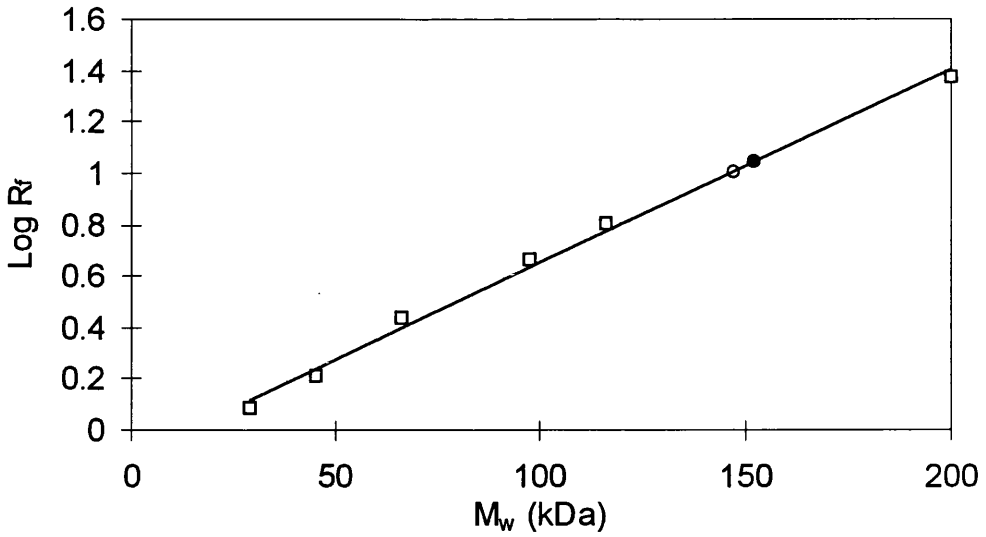


Fig. 5.2. Determination of the molecular weight of HMXOR and BMXOR by SDS-PAGE. As described in Section 5.2.1. Symbols represent; molecular weight standards (open squares), BMXOR (open circle) and HMXOR (filled circle).

5.3.2 Native-PAGE

HMXOR and BMXOR were run on native-PAGE according to the method described in Section 5.2.2. Fig. 5.3 shows the native gels with concentrations of 5%, 6%, 7% & 8%. R_f and K_R values were calculated and plotted as described in Section 5.2.2 (Fig. 5.4), and the molecular weights estimated (Table 5.1).

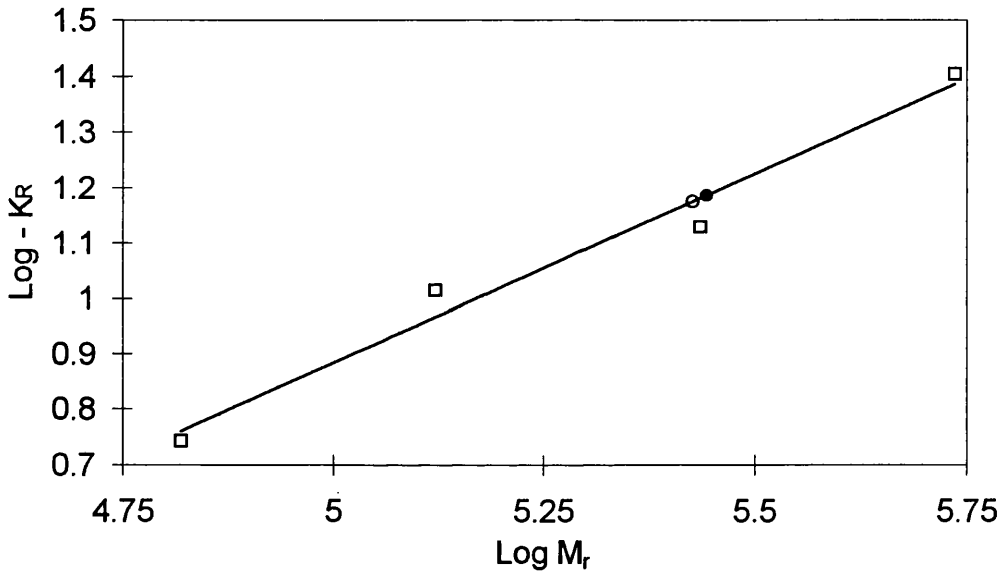


Fig. 5.4. Determination of the molecular weights of HMXOR and BMXOR by Native-PAGE. As described in Section 5.2.2. Symbols represent; non-denaturing high molecular weight standards (open squares), BMXOR (open circle) and HMXOR (filled circle).

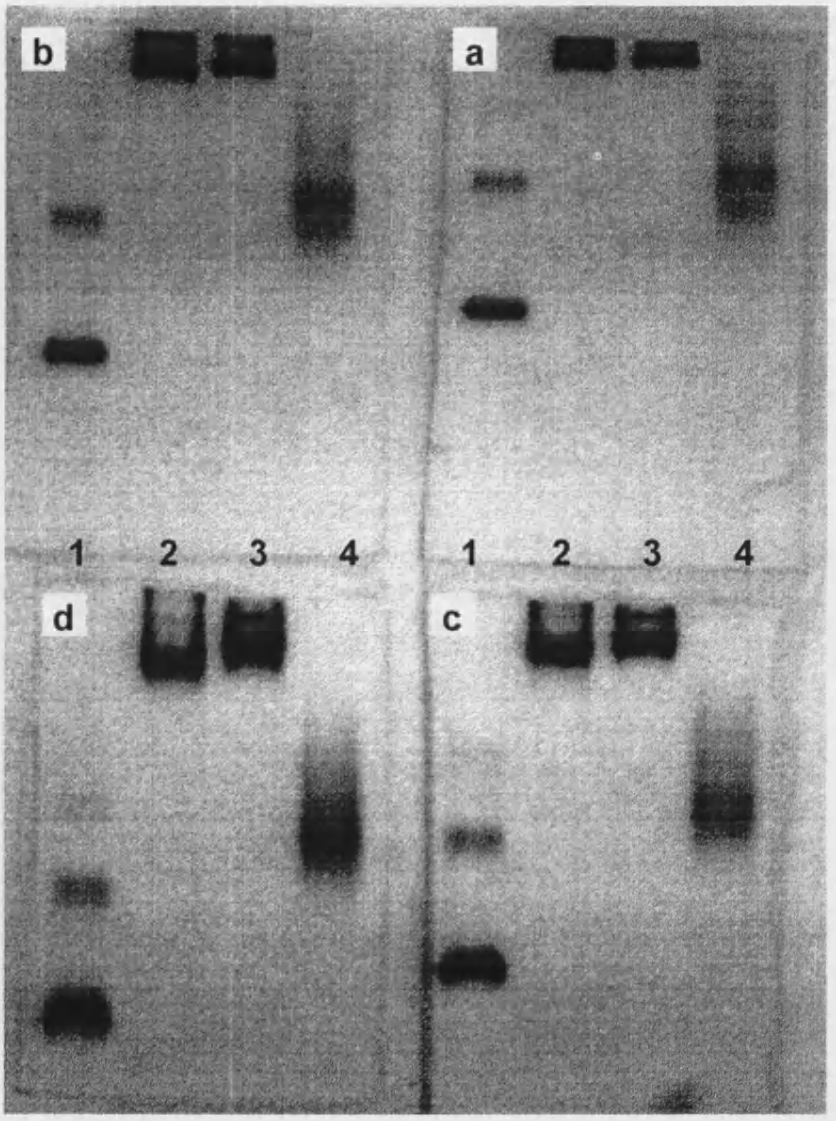


Fig. 5.3. Native-PAGE of HMXOR and BMXOR. HMXOR and BMXOR preparations were run on 5%, 6%, 7% and 8% Native-PAGE gels (Section 5.2.1) and stained for protein (Section 5.2.1). Gels a, b, c and d, correspond to 5%, 6%, 7% and 8% gels respectively. Lanes 1 BSA; lanes 2, BMXOR; lanes 3, HMXOR and lanes 4, Alcohol dehydrogenase (yeast).

5.3.3 Gel filtration

Gel filtration was used to estimate the molecular weight of HMXOR and BMXOR as described in Section 5.2.3. Elution volume was plotted against log M_r (Fig. 5.5) and the molecular weights were estimated, (Table 5.1).

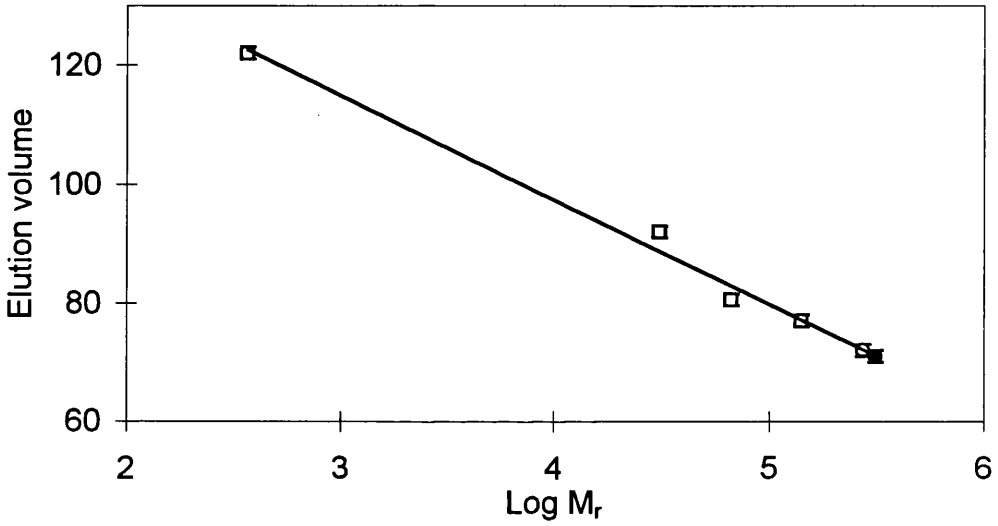


Fig. 5.5. Determination of the molecular weight of HMXOR and BMXOR by FPLC on Superdex 200. As described in Section 5.2.3. Symbols represent; ADH (141 kDa), BSA (66.2 kDa), CA (31 kDa) and DNP-lysine (367 Da) (open squares), BMXOR (open circle) and HMXOR (filled circle).

5.3.4 Dynamic Light Scattering

Dynamic light scattering was used to provide an estimation of Stoke's radius and hence molecular weight (see Section 5.1). HMXOR and BMXOR samples, purified from fresh human and bovine milk, were used. The data indicated that the polydispersity figures were sufficiently low to suggest that samples were virtually monodisperse. Unlike other samples of HMXOR used in light scattering studies the enzyme used here, purified from fresh human milk in the presence of 10 mM DTT, was found to be virtually monodisperse. The results are shown in table 5.1.

	HMXOR	BMXOR
SDS PAGE	151 kDa (±2 kDa)	147 kDa (±2 kDa)
Native PAGE	277 kDa (±6 kDa)	267 kDa (±6 kDa)
Gel Filtration	311 kDa (±16 kDa)	273 kDa (±16 kDa)
Light Scattering	259 kDa (±8 kDa)	243 kDa (±1 kDa)

Table 5.1 Molecular weights determined by SDS-PAGE, native-PAGE, gel filtration and dynamic light scattering. As determined in Sections 5.3.1, 5.3.2, 5.3.3 and 5.3.4.

5.3 Discussion

The methods of estimating molecular weights, described here, consistently show slightly higher vales for HMXOR than for BMXOR. These findings contrast with those recently derived from MALDI-TOF mass spectrometry (R. C. Bray, personal communication) whereby values of 148 ± 0.7 kDa (n=12) and 148.3 ± 0.5 kDa (n=6) were obtained for HMXOR and BMXOR respectively. The amino acid sequence of BMXOR has been reported (Berglund *et al.*, 1996, see Appendix Section 12.1) and is consistent with a molecular weight, including a full complement of cofactors, of 148.3 kDa; clearly in excellent aggreement with MALDI-TOF MS. The primary sequence of human liver XOR (HLXOR) has also been reported (Ichida *et al.*, 1993, see Appendix Sections 12.1) and, although having 143 amino acid changes, is of essentially the same size. HMXOR has been sequenced (Briggs & Pearson, personal communication, see Appendix Section 12.3) apart from approximately 200 N-terminal residues and it is, so far, essentially identical to HLXOR.

It appears, therefore, that HMXOR and BMXOR could differ slightly in tertiary structure. HMXOR contains approximately 95% demolybdo form, compared with approximately 40% for BMXOR (Chapter 9) and, while the extent to which molybdenum cofactor contents parallel these percentages is not clear, it is possible that the higher content of demolybdo form in

HMXOR will be reflected in a more open conformation, with increased Stoke's radius. While this argument might serve to explain the results of native-PAGE, gel filtration and light scattering, it will not so serve for SDS-PAGE. Moreover, explanations based soley on an open conformation of demolybdo enzyme might predict more than one apparent value for BMXOR (40% demolybdo), which was never observed.

As noted above, differences in apparent molecular weight shown by SDS-PAGE, consistently observed in our laboratory over several years, cannot be explained in terms of tertiary structure. The possibility that HMXOR might be resistant to complete reduction and denaturation was considered and rejected following extended heating times in denaturing sample buffer (data not shown). It is known that a proline rich sequence can lead to low mobility on SDS-PAGE. Full comparison of amino acid sequences of HMXOR and BMXOR awaits data for the first 200 amino acids, apart from which the two enzymes are essentially identical. However, residues 193 - 225 of BMXOR in the loop region between the Fe-S and FAD domains (Sato *et al.*, 1995) contain 9 prolines in 32 residues and it will be interesting to compare the sequence of HMXOR in this region.

6 Aggregation Studies

6.1 Introduction

Aggregation of HMXOR has been noted as a problem since its first characterisation (Abadeh *et al.*, 1992). Visible aggregation has been observed after storage, during concentration or in thawing frozen solutions. Centrifugation to remove aggregated precipitates leads to loss of enzyme, the remaining solution having a relatively increased amount of soluble impurities. Aggregation is particularly a problem with HMXOR as it appears to aggregate more readily than BMXOR. This chapter describes attempts to characterise the nature of the aggregation observed with HMXOR.

Considerable effort was expended in attempts to use light scattering as a tool in these studies. Light scattering has been used to monitor the aggregation of proteins and other molecules (Yamamoto *et al*,. 1995; Asnaghi *et al.*, 1995). Light scattering has the advantages that it is non-destructive and requires small samples. Dynamic light scattering is based on the measurements of fluctuations in scattered light intensity caused by the relative movements of macromolecules in solution. The time scale of these fluctuations depends on the speed of movement of molecules moving under Brownian motion, allowing the translational diffusion

coefficient (D_T) to be obtained. Once D_T has been determined, the hydrodynamic radius (R_H) can be calculated by using the Stokes-Einstein equation. Molecular weights are then estimated using the relationship between molecular weight and R_H determined for a series of standard proteins. In tables derived from dynamic light scattering studies four values are quoted. First the molecular weight (kDa) as estimated from the second value, the radius (nm). The third value is polydispersity (nm), which is the standard deviation for the radius and the fourth value is the baseline value. This is a statistical parameter which, at best is 1, indicates the reliability of the data measured.

Where aggregates of many different sizes are present in the solution, the dynamic light scattering generated values of molecular weight give an average. The values obtained can be interpreted to give an estimation of aggregation, as polydispersity is seen to increase with the molecular weight.

6.2 Materials and methods

6.2.1 Native-PAGE

Native gels were prepared as described in Section 5.2.2 and stained for protein as described in Section 5.2.1 or activity by incubating at room temperature in 50 mM Tris HCl, pH 7.5, containing 20 mM xanthine and 3 mM nitroblue tetrazolium chloride (NBT), until bands appeared, usually 10-30 min (Waud & Rajagopalan, 1976 a).

6.2.2 Dynamic light scattering

XOR solutions (0.5 ml) in 50 mM Na-phosphate, pH 7.4, were injected into an Oros Instruments M801 Molecular Size Detector through a Whatman anotop 10 0.1 μm syringe filter using a 1 ml syringe. Data were handled using the Autopro software supplied.

6.3 Results

6.3.1 Gel Filtration

Aggregated HMXOR samples were loaded onto the gel filtration column and eluted at a flow rate of 1 ml/min, as described in Section 5.2.3. Fig. 6.1 shows the elution profile of HMXOR. The largest peak, peak 1 was calculated to have a molecular weight of 311 ± 16 kDa, corresponding to HMXOR. Peak 2 was estimated to have a molecular weight of approximately 700 ± 122 kDa, although determinations of molecular weights as high as this are inaccurate, and peak 3 was eluted in the void volume. SDS-PAGE on fractions from all three peaks confirmed the presence of a 150 kDa protein band; however xanthine oxidase activity could only be detected in peaks 1 and 2, possibly because the concentration of XOR protein in peak three was too low.

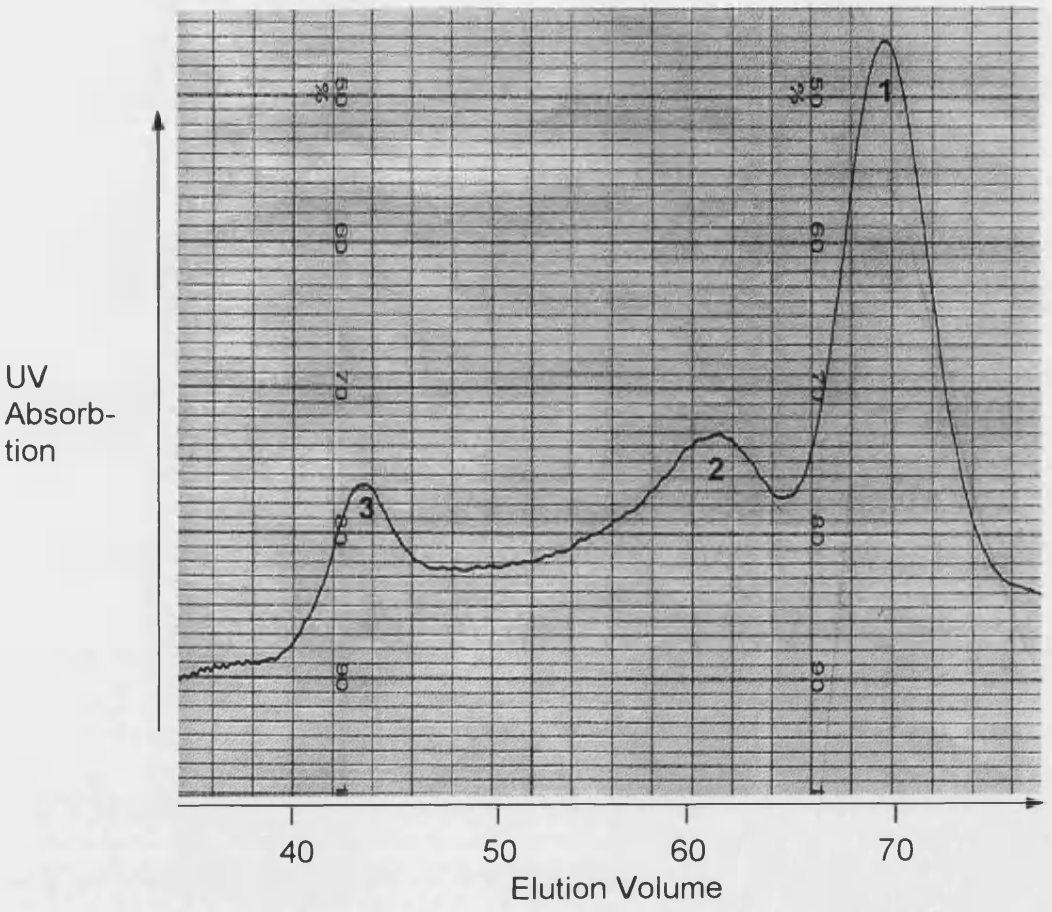


Fig. 6.1. FPLC elution profile of aggregated HMXOR from Superdex 200. An aggregated sample of HMXOR was eluted at 1 ml/min on a Superdex 200 column as described in Section 6.3.1. Peak 1 corresponds to native HMXOR dimer (≈300 kDa), peak 2 corresponds to a tetramer (≈700 kDa) and peak 3 is eluted in the void volume.

6.3.2 Native-Page

Samples of HMXOR were run on native-PAGE. The gels were separately stained for protein and enzymic activity (Sections 5.2.1 & 6.2.1). Both gels (Fig. 6.2) showed similar staining, with the majority of protein and activity running as a band on the gels corresponding to HMXOR (Section 6.3.2). Despite considerable smearing a fainter band with lower mobility was observed on both protein- and activity-stained gels.

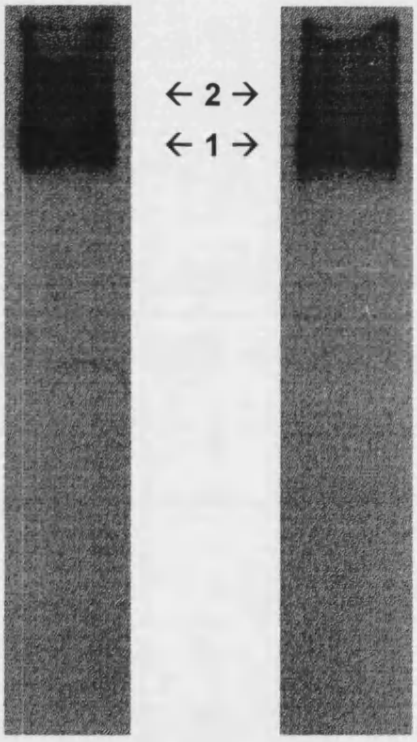


Fig. 6.2. Native-polyacrylamide gels (7.5%) of aggregated HMXOR. Aggregated HMXOR was subjected to native-PAGE and the gel stained for activity (left) and protein (right) (Sections 6.2.1 & 5.2.1). Band 1 corresponds to HMXOR with a molecular weight of 300 kDa. Band 2 corresponds to a much larger species (weight not determined).

6.3.3 Effect of concentration on aggregation of HMXOR

Freshly-prepared HMXOR and BMXOR solutions were diluted to concentrations of 1, 0.5 and 0.2 mg/ml, the last concentration being the lower limit of detection for the dynamic light scattering detector. 10 min after dilution, samples were injected into the detector for estimation of molecular weight. The results, (Table 6.1), indicate that HMXOR was largely aggregated at all three concentrations. However, the aggregation, as judged by both the estimated molecular weight and the polydispersity, decreased with dilution. In contrast, the similarly-prepared BMXOR sample appeared to be totally unaggregated even at the highest concentration.

Concentration (mg/ml)	Estimated Mr (kDa)	Radius (nm)	Polydispersity (nm)	Baseline
HMXOR				
1.0	47693	54.8	30.4	1.03
0.5	39994	50.9	28.3	1.04
0.2	33564	47.4	26.3	1.04
BMXOR				
2.0	292	6.7	1.7	1.00
0.5	282	6.6	1.8	1.01

Table 6.1. The effect of concentration on aggregation of HMXOR and BMXOR. HMXOR (1 mg/ml) was diluted to 0.5 mg/ml and 0.2 mg/ml and similarly prepared BMXOR, 2 mg/ml, was diluted to 0.5 mg/ml. The samples, 10 min after dilution, were subjected to dynamic light scattering for estimation of molecular weight as described in Section 6.2.2.

6.3.4 Effect of NaCl on aggregation of HMXOR

1M NaCl solution was added to HMXOR and BMXOR so as to achieve the final concentrations shown in Table 6.2. 10 min after addition, samples were injected into the detector for estimation of molecular weight. The results, (6.2), show a trend of decreasing aggregation with increasing NaCl concentration. Again, BMXOR shows little or no aggregation even in the absence of NaCl.

NaCl (M)	Estimated Mr (kDa)	Radius (nm)	Polydispersity (nm)	Baseline
HMXOR				
0.0	47693	54.8	30.4	1.03
0.1	31356	46.1	25.5	1.04
0.2	27529	43.7	24.2	1.04
0.5	14983	34.0	18.8	1.04
BMXOR				
0.0	292	6.7	1.7	1.00
0.1	273	6.5	1.6	1.00

Table 6.2. The effect of NaCl on aggregation of HMXOR and BMXOR.

To HMXOR (1 mg/ml) and to similarly-prepared BMXOR (2 mg/ml), NaCl was added to final concentrations of 0.1 M, 0.2 M and 0.5 M. The samples, 10 min after addition, were subjected to dynamic light scattering for estimation of molecular weight as described in Section 6.2.2.

6.3.5 Effect of dithiothreitol on aggregation of HMXOR

DTT was added to a sample of HMXOR and injected into the detector for estimation of molecular weight. As a control, a sample which had no DTT added was also injected. The molecular weight was monitored over time and the results are shown in Fig. 6.3. HMXOR, without DTT added, did not change significantly over the time-course. In the presence of DTT the apparent molecular weight was seen to decrease significantly and to approach that expected for unaggregated enzyme.

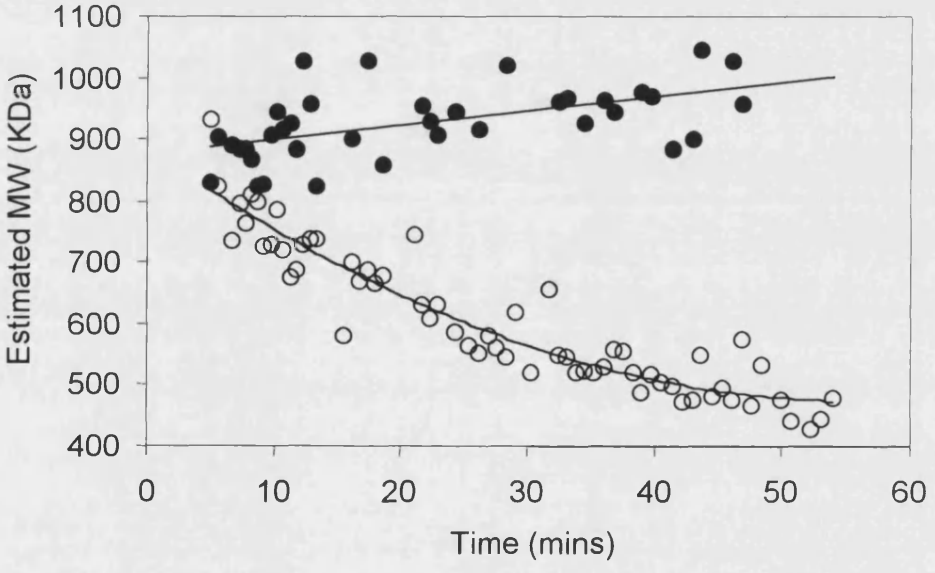


Fig. 6.3. The effect of DTT on aggregation of HMXOR. 10 mM DTT was added to aggregated HMXOR and the sample was injected into a dynamic light scattering molecular weight detector. The molecular weight was estimated over time (**O**). The same sample without DTT was also monitored (●) which showed a slight increase in molecular weight over the time-course.

6.3.6 Activity of aggregated enzyme

A preparation of HMXOR displaying visible turbidity was centrifuged (13000g, 10 min.) and the pellet was redissolved in buffer containing 10 mM DTT. Table 6.3 shows that the original sample, the centrifugate and the supernatant all had similar xanthine oxidase specific activities.

	Volume (μl)	Protein (mg)	Specific Activity (μmol $min^{-1}mg^{-1}$)
HMXOR sample (62% Oxidase)	500	0.24	62.0 ± 6.0
supernatant after centrifugation	500	0.19	58.0 ± 6.3
pellet after resuspending in DTT	130	0.06	70.0 ± 4.2

Table 6.3. Xanthine oxidase activity of aggregated and soluble HMXOR. A sample of HMXOR in 50 mM Na Bicine, pH 8.3, that was visibly turbid was centrifuged (13000g 10 min). The resulting supernatant was decanted and the pellet was dissolved in 50mM Na Bicine, pH 8.3, containing 10mM DTT. For each of the three fractions xanthine oxidase activities (Section 3.2.3) and protein concentrations (Section 3.2.1) were measured and the specific activity calculated.

6.4 Discussion

Gel filtration and native PAGE clearly show the presence of aggregates in samples of HMXOR, and the aggregated enzyme is showed to be enzymically active. HMXOR used in these experiments contained largely the demolybdo form (Chapter 10), which in the case of BMXOR has been more susceptible to denaturation under certain conditions (Bergel & Bray, 1956; Ventom, *et al.*, 1988). HMXOR in the present study was clearly more prone to aggregation than BMXOR, however, the aggregates appeared to be enzymically active and not denatured, as evidenced by gel filtration and native-PAGE. Moreover, aggregated HMXOR, removed by centrifugation, had a similar specific activity to that of soluble HMXOR.

The greater susceptibility to aggregation of active HMXOR, compared to BMXOR, presumably reflects some structural or charge differences between enzyme from the two sources. The essentially predictable effects of concentration and NaCl on aggregation of HMXOR provide few clues. The disaggregating effect of DTT, unlike that of NaCl, was time-dependant on a time scale with that of oxidase to dehdrogenase conversion (Chapter 8) suggesting that differences in aggregatability between HMXOR and BMXOR may be primarily confined to the oxidase form.

7 Spectral Analyses

7.1 Introduction

Because of the chromophores present in XOR, UV visible spectra can reveal much about the composition of the enzyme and also about functional differences between its different forms (Hille & Nishino, 1995). On a more practical level, a spectrum of a given sample of enzyme can allow an accurate determination of concentration and purity (Avis *et al.*, 1955). In preliminary studies, significant differences were observed between the spectra of HMXOR and BMXOR and these were investigated in the presently described work. In so far as all three chromophores of XOR, iron-sulphur, FAD and the molybdenum centre, are optically active, circular dichroism (CD) lends itself to study of the enzyme, although only a few relevant publications have appeared (Garbett *et al.*, 1967; Palmer & Massey, 1969; Bayer *et al.*, 1971; Hunt & Massey, 1992). In the present comparison of HMXOR and BMXOR, CD has proved to be particularly informative.

7.2 Materials and Methods

7.2.1 Flavin estimations

To determine the $\in_{450}$ of HMXOR and BMXOR preparations, the flavin fluorescence of the enzyme in 5% trichloracetic acid was compared to that of standard solutions of flavin. Enzyme samples in 50 mM phosphate buffer, pH 7.4, containing 5% TCA were incubated at 4°C for 30 min and centrifuged at 13000g for 10 min at 4°C. The flavin fluorescence at 525 nm was measured with excitation at 450 nm in a Perkin-Elmer LS-5B Luminescence Spectrometer, and FAD was quantified ($\in_{450}$ = 11300M^{-1} cm^{-1}) by comparison with standard solutions of FAD.

7.2.2 UV-visible spectra

UV-visible spectra of enzyme samples in 50 mM phosphate buffer, pH 7.4, were recorded using a Cecil CE 6600 Multimode Computing UV Spectrophotometer. Data were collected by an IBM compatible personal computer using software designed and written by Dr R. Maytum.

7.2.3 CD spectra

CD spectra of enzyme samples in 25 mM potassium phosphate, pH 7, were recorded by Dr. G Siligardi at the EPSRC National Chiroptical Spectroscopy & ULIRS Optical Spectroscopy Centres, King's College London. Far-UV and UV-visible CD spectra were recorded using a Jasco J-600 and Jasco J-700 CD spectrophotometers respectively.

7.3 Results

7.3.1 Molar Extinction Coefficients

The flavin content of a sample of HMXOR and BMXOR and three samples of demolybdo BMXOR were determined (Section 7.2.1) and, on the assumption that each subunit contains its full complement of FAD, ϵ_{450} values were calculated from the UV-visible spectra. These values, shown in Table 7.1, can be compared with that of 36000M^{-1} cm^{-1} quoted by Bray (1975) for BMXOR containing its full complement of cofactors. Although, in my hands, BMXOR showed a value similar to that of Bray, the molar extinction coefficient for HMXOR was considerably lower at 28500 M^{-1} cm^{-1}. Demolybdo BMXOR prepared by folate affinity chromatography (Section 4.3.5) showed values intermediate between those of HMXOR and BMXOR.

Sample and Preparation Number	A450 M FAD^{-1}
HMXOR 1	28500 ± 1300
BMXOR 2	34800 ± 1200
BMXOR 4 demolybdo	35000 ± 1600
BMXOR 5 demolybdo	33300 ± 700
BMXOR 6 demolybdo	28600 ± 1100

Table 7.1. Calculations of molar extinction coefficients from FAD estimations. FAD was estimated as described (Section 7.2.1) and used to calculate the ϵ_{450} of HMXOR, BMXOR and three demolybdo BMXOR preparations.

7.3.2 UV-visible Spectra

UV-visible spectra of HMXOR, BMXOR and demolybdo BMXOR 6 were measured and replotted at identical molarities, according to the FAD content (Fig. 7.1). The difference spectrum of HMXOR subtracted from BMXOR gave a spectrum similar to those of the non-haem Fe-S containing proteins (Shethna *et al.*, 1968) (Fig. 7.1, inset), suggesting that HMXOR lacks Fe-S.

This apparent Fe-S deficiency was estimated from the extinction coefficients by using the following equation, based on the assumptions that enzyme with an ϵ_{450} 36000 M^{-1} cm^{-1} has 100% Fe-S content and that the absorbance at 450 nm is solely due to FAD and Fe-S.

% Deficiency = $[1-(\epsilon_M$ Sample - ϵ_M FAD) / (ϵ_M BMXOR - ϵ_M FAD)] $\times 100$

Where ϵ_M BMXOR = 36000 M^{-1} cm^{-1}, and ϵ_M FAD = 11300 M^{-1} cm^{-1}

The results of these calculations are summarised in Table 7.2.

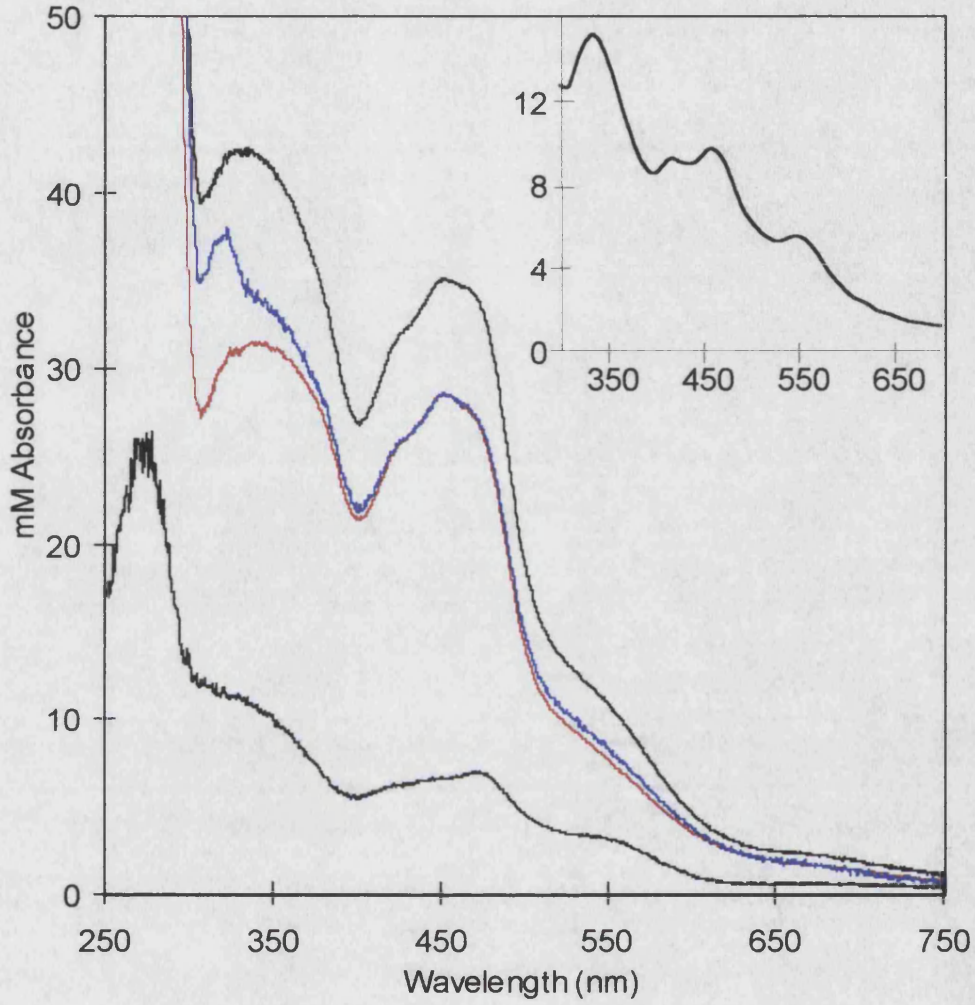


Fig. 7.1. Near UV-visible absorption spectra of BMXOR, HMXOR and demolybdo BMXOR. Spectra of BMXOR (———); HMXOR (———); demolybdo-BMXOR (———) were taken at concentrations of 8.9, 8.1 and 3.1 μM respectively and replotted using the $\in_{450}$ values in Table 7.1 to normalise them on the basis of FAD content. The lower curve is the difference spectrum after subtraction of the HMXOR spectra from the BMXOR spectra. Inset; Fe-S protein I (Shethna *et al.*, 1968).

	UV-Visible Data		CD Data *	
Sample	Fe-S/ FAD	% Fe-S Deficiency	Fe-S/ subunit	% Fe-S Deficiency
BMXOR 2	1.90	4.9	2.00	0.0
HMXOR 1	1.39	30.4	1.38	31.3
Demolybdo BMXOR 6	1.40	30.0	1.42	28.8

Table 7.2. Estimation of Fe-S/FAD ratios, and % Fe-S deficiencies from the UV-visible and CD spectra. Molar extinction coefficients (Table 7.1) were used to calculate the Fe-S content and deficiencies from the UV-visible spectrums as described in Section 7.2.2. CD 342 nm molar extinction coefficients were used to calculate the Fe-S content and deficiencies as described in Section 7.3.4. * These data assume an Fe-S/FAD of 2 for BMXOR.

7.3.3 Simulation of the UV-visible spectrum of HMXOR

If we accept that HMXOR is 30% deficient in Fe-S (Table 7.2) then it should be possible to simulate the HMXOR spectra by addition of FAD spectra to the BMXOR spectra in such proportions as to recreate the ratio Fe-S/FAD of HMXOR. Fig. 7.2 shows the spectra of HMXOR, BMXOR + FAD and the difference spectrum. It can be seen that the simulated HMXOR spectrum agrees very well with that of HMXOR itself. Fig. 7.3 shows spectra matched in terms of their $\in_{450}$ values, clearly a much worse fit.

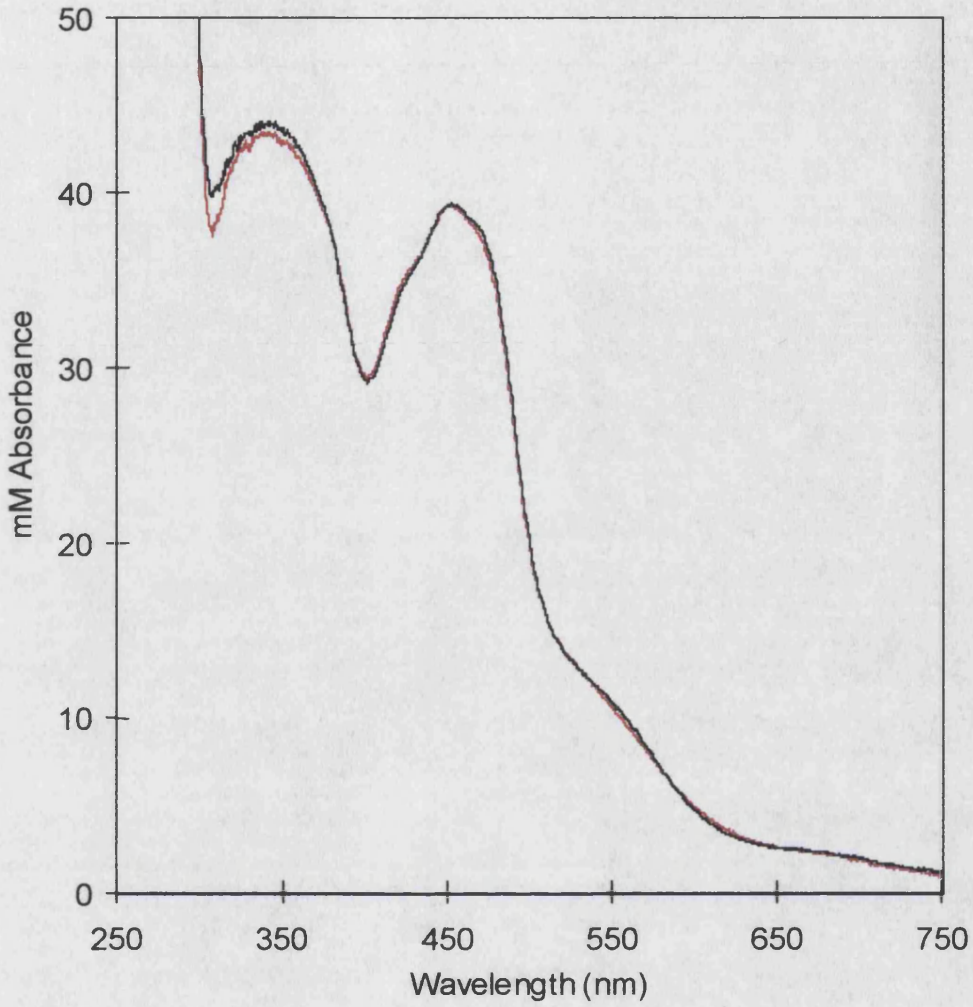


Fig. 7.2. Near UV-visible absorption spectra of HMXOR and simulated HMXOR. The spectra of HMXOR was simulated by computed addition of the spectra of BMXOR and FAD to give the same Fe-S/FAD ratio as HMXOR (Table 7.2), as described in Section 7.3.3, (———), and plotted with the appropriately matched spectra of HMXOR (———).

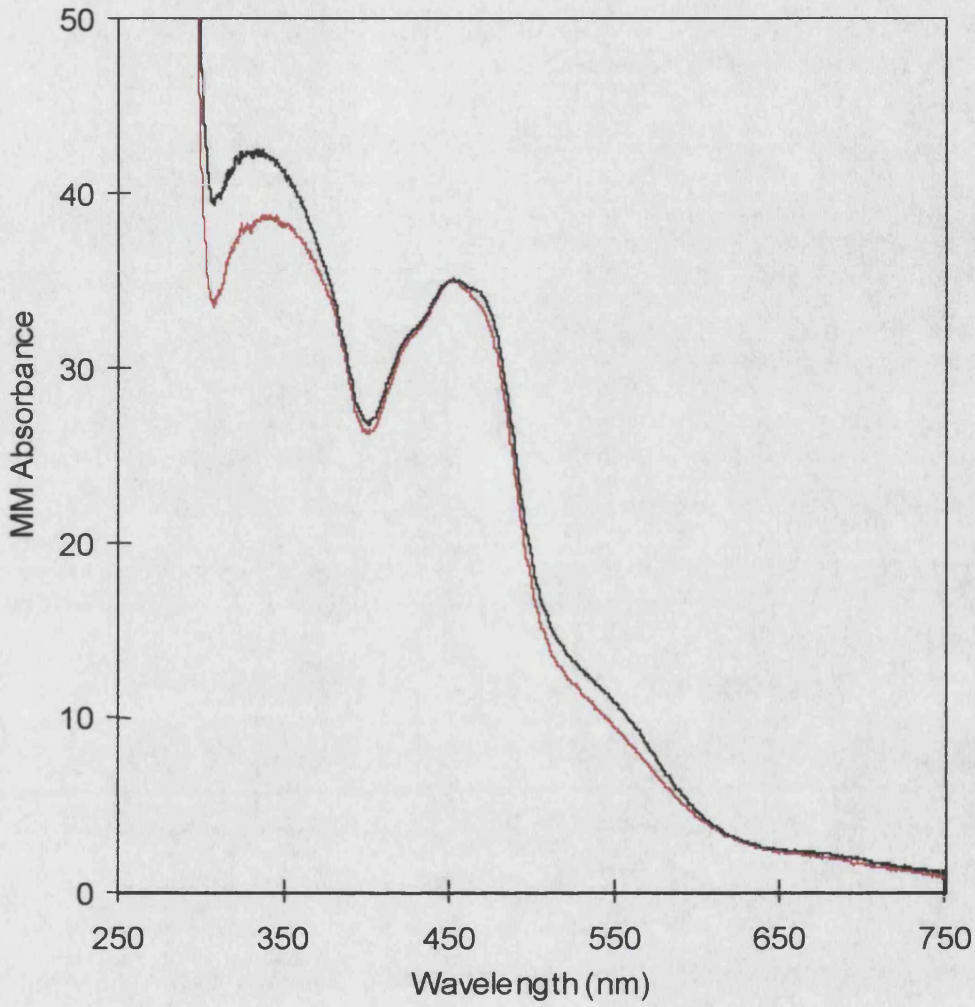


Fig. 7.3. Near UV-visible absorption spectra of HMXOR and BMXOR. The molar absorption spectra of BMXOR and HMXOR were replotted after that of HMXOR (———) had been appropriately matched at 450nm to that of BMXOR (———).

7.3.4 CD Spectra

Near-UV-visible CD spectra of HMXOR, BMXOR and demolybdo BMXOR 6, were measured, and replotted at identical molarities according to their $\in_{450}$ values (Table 7.1). As can be seen in Fig. 7.4, above 350 nm, whereas all three spectra show essentially the same form, their intensities differ, with BMXOR showing markedly greater Cotton effects than HMXOR or demolybdo BMXOR 6. Spectra of the latter two species are remarkably similar. These CD spectra derive very largely from the Fe-S centres (Bayer *et al.*, 1971) and provide further evidence of a deficiency of Fe-S centres for HMXOR and demolybdo BMXOR relative to BMXOR. CD $\in_{432}$ values for HMXOR, BMXOR and demolybdo BMXOR 6 of 49.5, 34.0 and 35.2 respectively were determined from the spectra and used to calculate Fe-S deficiencies assuming that BMXOR has a full complement of Fe-S and that Fe-S centres were solely contributing to the CD spectra at this wavelength (Table 7.2). If the CD spectra are normalised, replotting the data using the apparent Fe-S deficiency (Table 7.2) then, above 350 nm, all three spectra are much more similar (Fig. 7.5). Relatively small differences are still, however apparent, particularly in the 200-400 nm region as can be seen from Fig. 7.5. Other small differences in the 450 to 750 region are highlighted in Fig. 7.6 which is a difference CD spectrum of HMXOR, after normalising for Fe-S, with BMXOR subtracted.

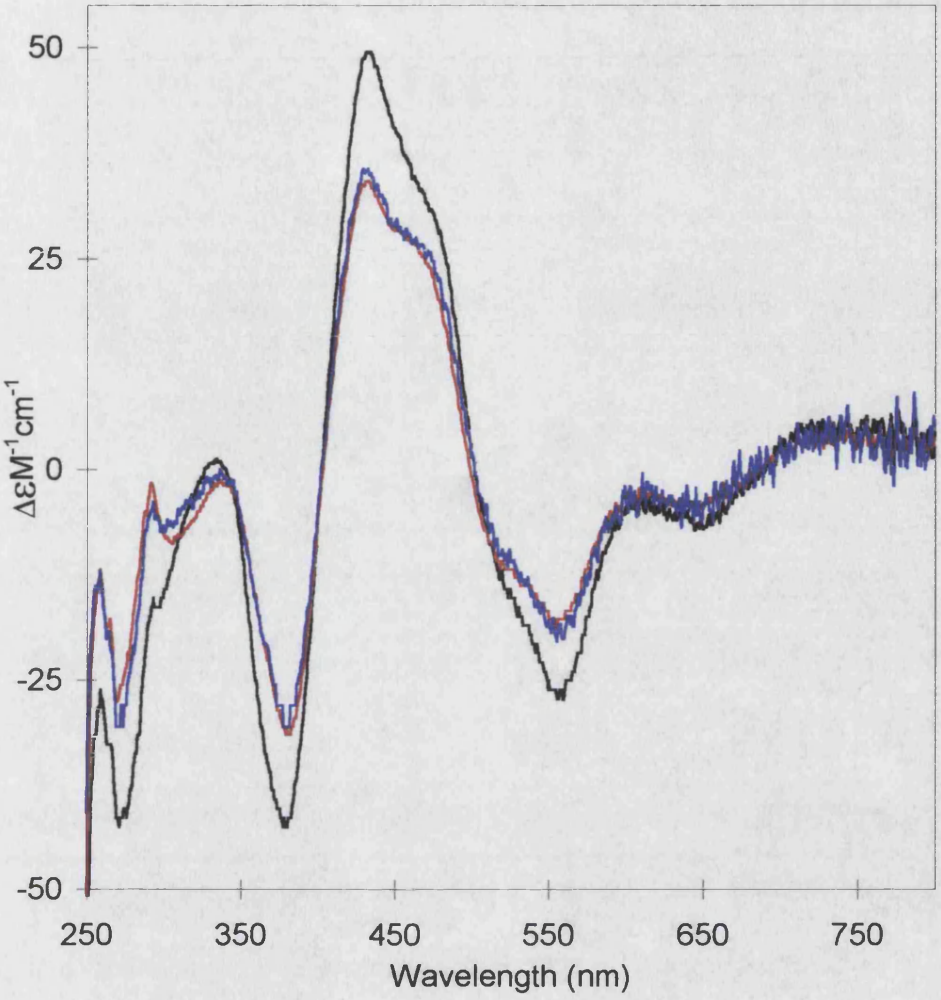


Fig. 7.4. Near UV-visible molar CD spectra of HMXOR (———), BMXOR (———) and demolybdo BMXOR 6 (———). CD spectra were determined and replotted using the near UV-visible molar absorption spectra as the reference.

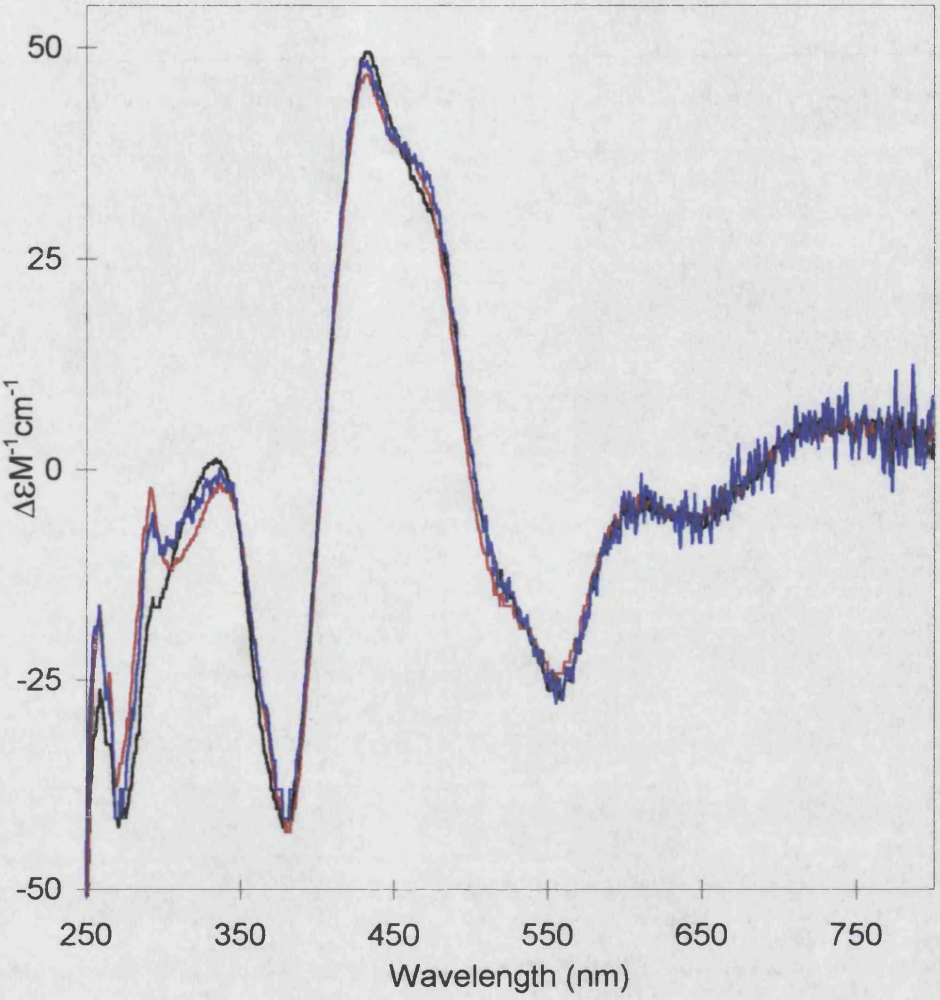


Fig. 7.5. Near UV-visible CD spectra of HMXOR (———), BMXOR (———) and demolybdo BMXOR 6 (———) after normalisation for Fe-S content. The molar CD spectra (Fig. 7.4) were replotted to normalise the Fe-S content (Table 7.2 column 1) to eliminate gross differences observed due to the observed Fe-S deficiency of HMXOR and demolybdo BMXOR.

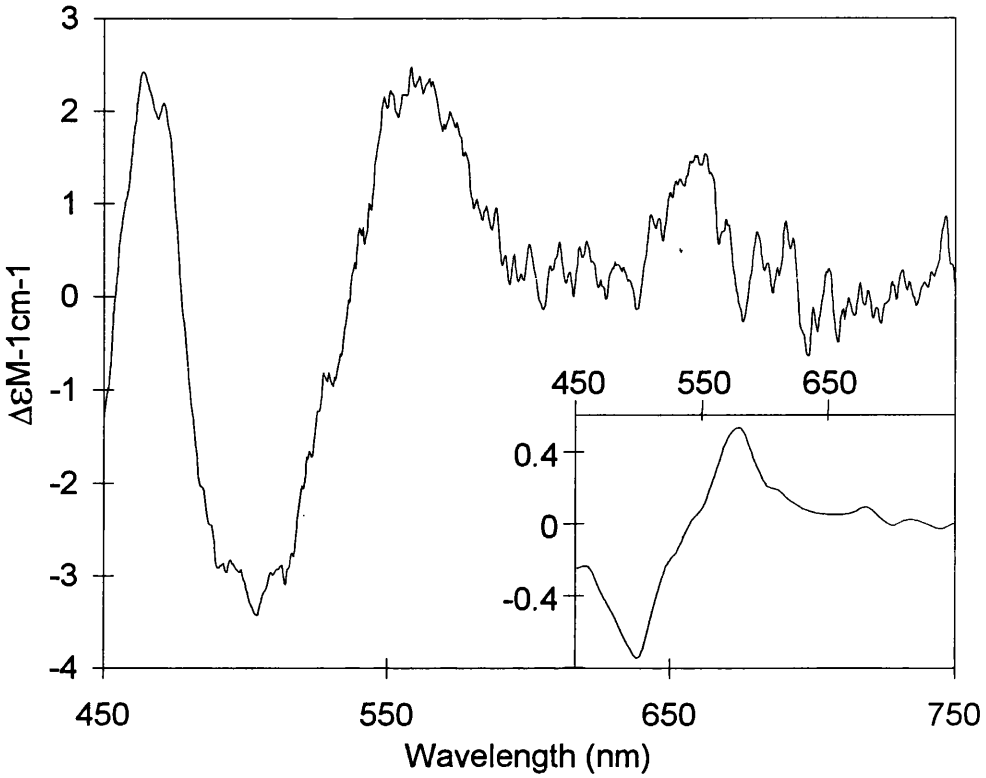


Fig. 7.6. Near UV-visible CD difference spectra of BMXOR and HMXOR after elimination of gross Fe-S deficiency. The difference CD spectum was obtained after subtraction of BMXOR from the HMXOR CD spectra in Fig. 7.5, to provide a non Fe-S HMXOR - BMXOR difference spectra. Inset shows the reported Mo^{IV} - Mo^{VI} difference CD spectrum (Ryan *et al.* 1995).

The far UV CD spectra of these samples was also measured, as shown in Fig. 7.7. These spectra, normalised for molarity, based on the extinction coefficients, are almost identical, indicating that the secondary structure content is essentially identical in the BMXOR, HMXOR and demolybdo BMXOR samples.

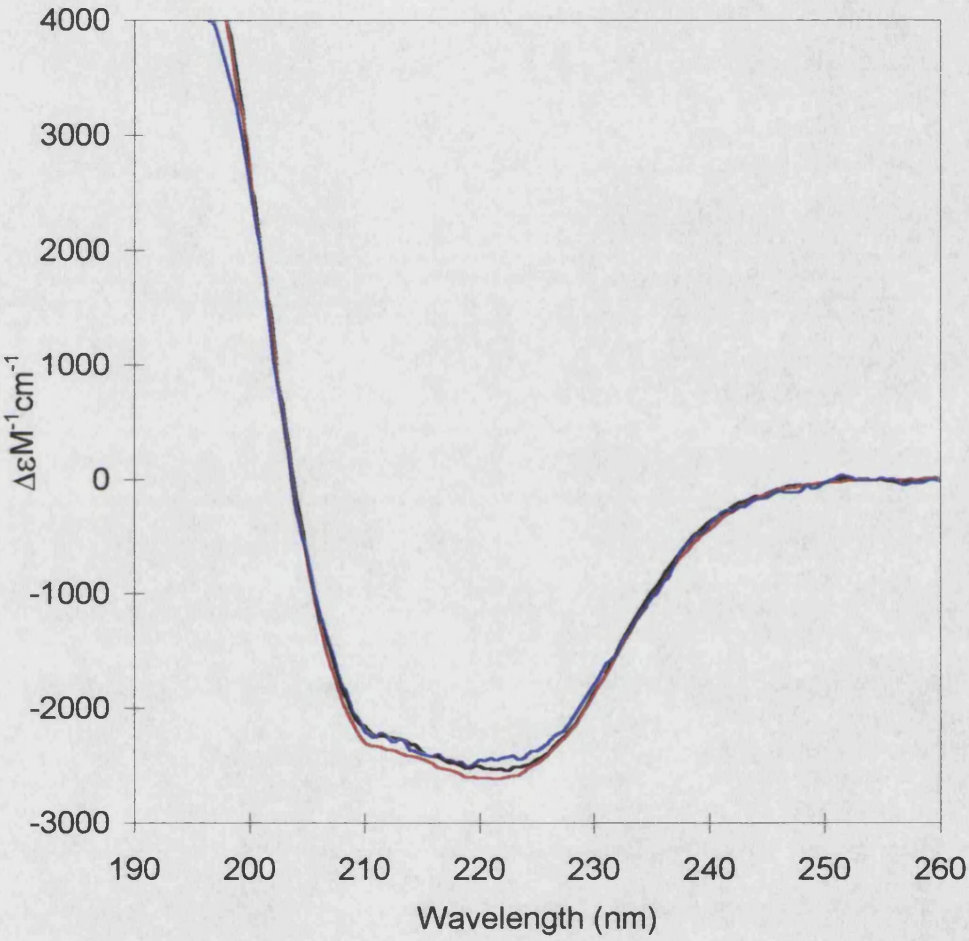


Fig. 7.7. Far UV molar CD spectra of BMXOR (———), HMXOR (———) and demolybdo BMXOR (———). Far UV CD spectra were taken and plotted using the near UV-visible molar absorption spectra as the reference.

7.4 Discussion

UV-Visible spectra of HMXOR and BMXOR, normalised on the basis of independently-measured FAD content, were found to differ significantly (Fig. 7.1), notably in their molar extinction coefficients at 450nm (Table 7.1). Not surprisingly, samples of demolybdo BMXOR, individually prepared by folate-affinity chromatography (Section 4.3.5) and differing in their molybdenum contents, differed also in their UV-visible spectra and extinction coefficient at 450nm (Table 7.1). Nevertheless, one such example, demolybdo BMXOR 6, showed a UV-visible spectrum very similar to that of HMXOR and a correspondingly similar extinction coefficient (Table 7.1).

Difference spectra (BMXOR - HMXOR) (Fig. 7.1) showed a spectrum similar to that shown by Fe-S alone suggesting that HMXOR is deficient in Fe-S, relative to BMXOR, by approximately 30% (Table 7.2). As FAD and Fe-S are the major contributors to the UV-visible spectrum, it was decided to simulate the Fe-S/FAD ratio of HMXOR by computed addition of FAD to the BMXOR spectrum. As can be seen in Fig. 7.2, this led to a remarkable fit with the HMXOR spectrum. Small residual differences in the 300 - 400 nm region were noted and may be attributable to the molybdenum cofactor which has been shown to have absorbance in this region with a

maximum at 350 nm (Johnson *et al.*, 1979). Other small residual differences could be attributable to the molybdenum.

The CD spectrum between 360 - 600 nm of BMXOR is known to result largely from the Fe-S groups (Bayer *et al.*, 1971) and this is clearly reflected in the spectrum of HMXOR (Fig. 7.4), which shows an extinction coefficient at 432 nm of 34.0M^{-1} cm^{-1}, compared with 49.5M^{-1} cm^{-1} for BMXOR; values that would be consistent with a Fe-S deficiency of approximately 30% for HMXOR. The CD spectrum of the demolybdo sample, BMXOR 6, was very similar to that of HMXOR, having an extinction coefficient at 432 nm of 35.2 M^{-1} cm^{-1}, consistent with a Fe-S deficiency of 29% (Table 7.2). These values of Fe-S deficiency for HMXOR and demolybdo BMXOR relative to BMXOR are remarkably consistent with those obtained from UV-visible spectra (Table 7.2). The CD spectrum of BMXOR (Fig. 7.4) is very similar to those published by Palmer & Massey (1969) and Gardlik *et al.* (1987), although the extinction coefficient (432 nm) quoted by Palmer & Massey is somewhat lower at 48 M^{-1} cm^{-1}, whereas that quoted by Gardlik *et al.* is higher at 52 M^{-1} cm^{-1}. The CD extinction coefficient for BMXOR after, allowance is made for Fe-S deficiency (Table 7.2, column 1), is 51.9 M^{-1} cm^{-1} (Fig. 7.5) which fits very well with that of Gardlik *et al.*.

When the CD spectra of HMXOR and BMXOR were normalised on the basis of Fe-S values (Table 7.2), fits were good (Fig. 7.5), although

significant differences remain, as evidenced by the difference spectrum shown in Fig. 7.6. It is tempting to attribute these differences to FAD, the content of which would be relatively lower in the spectrum of BMXOR after normalisation. However, above 300 nm, FAD shows only a weak Cotton effect at 375 nm, with extinction coefficient of -0.32 M^{-1} cm^{-1}, and negligible effects at higher wavelengths (Bayer *et al.* 1971; Siligardi, personal communication). It may be that differences in molybdenum content contribute to minor variations in the CD spectra above 350 nm. In this region, the CD spectrum of HMXOR is very similar to that of demolybdo BMXOR 6 (Fig. 7.4). Ryan *et al.* (1995) reported differences in CD spectra following reduction Mo^{IV} to Mo^{VI} and the corresponding difference spectrum (Fig. 7.6, inset) shows similarity to that for HMXOR - BMXOR (Fig. 7.6) above 430 nm.

Marked difference in the CD spectra of HMXOR, BMXOR and demolybdo BMXOR 6 are seen in the 300 - 350 nm region (Fig. 7.5). Cotton effects in this region have been attributed to the molybdenum cofactor (Gardlik *et al.* 1987) and indeed, Form A, one of its derivatives, shows a positive Cotton effect with a broad peak from 300 to 350 nm (Taylor *et al.*, 1989). Differences in molybdenum cofactor content may, accordingly, explain differences, in intensities of the Cotton effects at 325 nm, between the three different enzymes.

8 Oxidase and Dehydrogenase Forms of XOR

8.1 Introduction

It is known that human liver and milk XOR display the same general XDH to XO conversion characteristics as bovine milk and rat liver enzyme as very highly dehydrogenase form HMXOR can be obtained by incubation with DTT, and almost exclusively XO via trypsinisation (Abadeh *et al.*, 1992; Sanders *et al.*, 1997) (see General Introduction). The production of non-proteolysed human XO and the non-proteolytic interconversions have not been described. Here the reversible interconversions of HMXOR are described in detail, with comparisons to the bovine milk enzyme. The methods that are used here have been shown to be successful in interconversions with the bovine milk and rat liver enzymes.

8.2 Materials and Methods

8.2.1 XO to XDH Conversion using Dithiothreitol

Samples of HMXO and BMXO were incubated at 37°C in Na-Bicine, pH 8.3, containing 10 mM Dithiothreitol (DTT). Samples were taken regularly and assayed for xanthine oxidase activity in the presence and absence of NAD (Sections 3.2.3, 3.2.4). Xanthine oxidase activities were calculated

and converted to specific activities and the percent oxidase activity was calculated (Section 3.2.4).

8.2.2 XDH to XO Conversion using Molecular Oxygen

Samples of HMXDH and BMXDH, previously converted with DTT, were incubated at 37°C in air-equilibrated Na-Bicine, pH 8.3. Samples were taken regularly and assayed for xanthine oxidase activity in the presence and absence of NAD (Sections 3.2.3, 3.2.4). Xanthine oxidase activities were calculated and converted to specific activities and the percent oxidase activity was calculated (Section 3.2.4).

8.2.3 XDH to XO Conversion using DTDP

HMXDH and BMXDH was incubated at 25 °C for 1 hour in 50 mM Na-bicine, pH 8.3, containing 1 mM 4,4 dithiodipyridine (DTDP). Samples before and after incubation were assayed for xanthine oxidase activity in the presence and absence of NAD (Sections 3.2.3, 3.2.4) and the percent oxidase activity was calculated (Section 3.2.4).

8.2.4 Determination of free sulphydryl groups

HMXOR samples were incubated at 25°C in 50 mM Na-bicine, pH 8.3, containing 1 mM DTDP. The increase in absorbance at 324 nm was measured and the 4-thiopyridone concentration was calculated by an $\square_{325}$ 19800 M^{-1} cm^{-1} (Grassetti & Murray, 1967).

8.2.5 XDH to XO Conversions Using Oxidised Glutathione

BMXDH and HMXDH were incubated for 2h at 25°C in Na-bicine, pH 8.3, containing 10 mM oxidised glutathione. Samples before and after incubation were assayed for xanthine oxidase activity in the presence and absence of NAD (Sections 3.2.3, 3.2.4) and the percent oxidase activity was calculated (Section 3.2.4).

8.2.6 Heparin Affinity of HMXDH and HMXO

Samples of HMXOR were applied to a heparin agarose column (3 ml) and run on FPLC at a flow rate of 1 ml/min. Fractions were collected and the absorbance at 450 nm measured or assayed for xanthine dehydrogenase activity in the presence and absence of NAD (Sections 3.2.3, 3.2.4).

8.3 Results

8.3.1 XO to XDH Conversion using Dithiothreitol

Incubation of XO in 10 mM DTT caused the conversion to XDH. The time-scale of the conversions are shown in Figs. 8.1 and 8.2. The overall rate of conversion to HMXO and BMXO was similar. However, whereas the total specific activity for BMXOR decreased slightly during the conversion, the total specific activity of HMXOR was seen to increase.

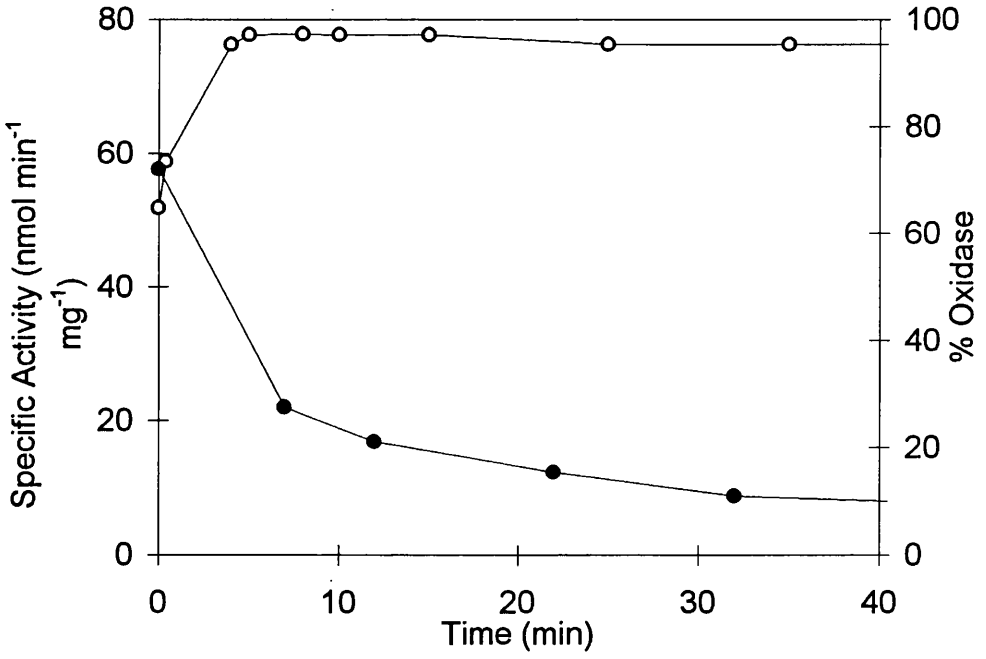


Fig. 8.1 Conversion of HMXO to HMXDH by treatment with DTT. HMXO was incubated with an excess of DTT at 37°C as described in Section 8.2.1. Enzyme was assayed over the incubation and the specific xanthine oxidase activity and percentage of oxidase activity calculated. (O) total xanthine oxidase activity, (●) percentage oxidase activity.

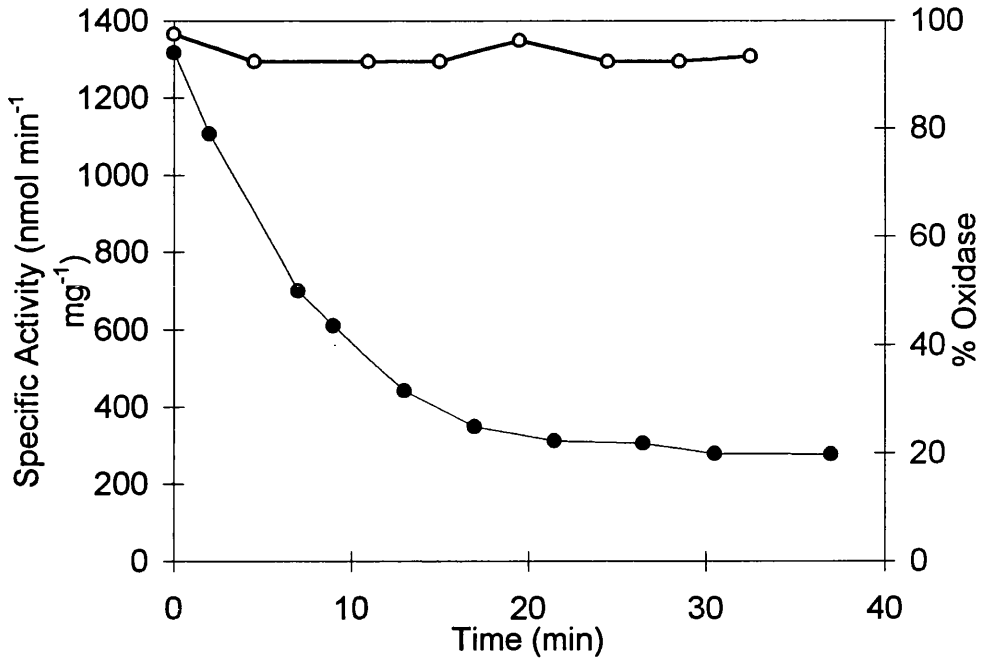


Fig. 8.2 Conversion of BMXO to BMXDH by treatment with DTT. BMXO was incubated with an excess of DTT at 37°C as described in Section 8.2.1. Enzyme was assayed over the incubation and the specific xanthine oxidase activity and percentage of oxidase activity calculated. (O) total xanthine oxidase activity, (●) percentage oxidase activity.

8.3.2 XDH to XO Conversion using Molecular Oxygen

XDH samples were incubated in air-equilibrated buffer at 37°C. The time-course data are shown in Figs. 8.3 and 8.4. The oxidase conversions of BMXOR and HMXOR were markedly different, the rate of conversion of HMXOR was approximately 10 times slower and over the time-course the % oxidase activity of HMXOR increased to less than 50%. The total

activity of BMXO increased slightly over the time course, whereas that for HMXO decreased considerably.

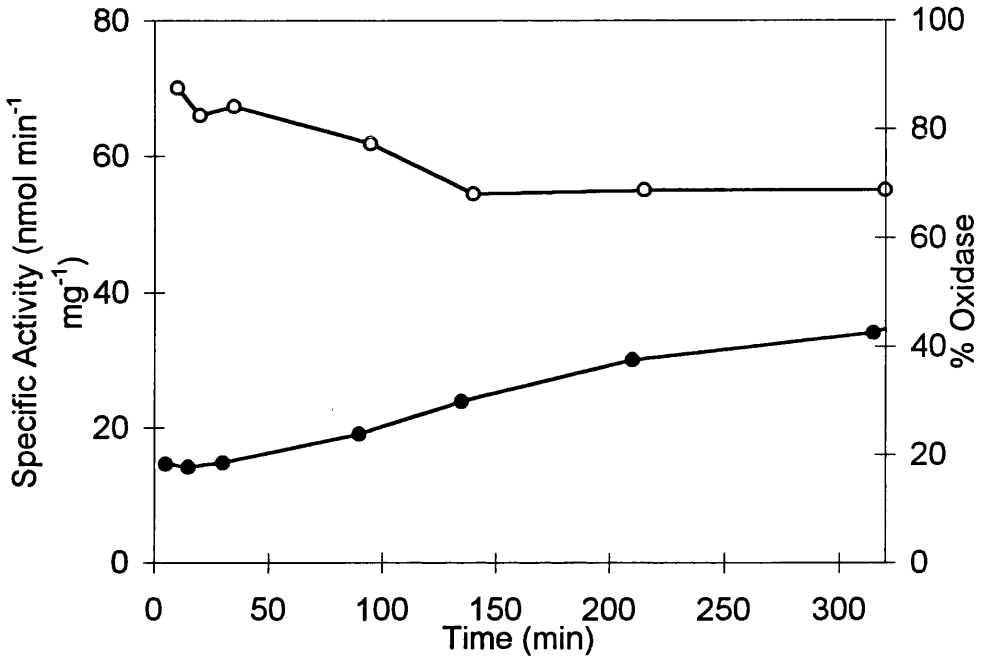


Fig. 8.3 Conversion of HMXDH to HMXO by incubation in air-equilibrated buffer at 37°C. HMXO was incubated in air equilibrated buffer at 37°C as described in Section 8.2.2. Enzyme was assayed over the incubation and the specific xanthine oxidase activity and percentage of oxidase activity calculated. (O) total xanthine oxidase activity, (●) percentage oxidase activity.

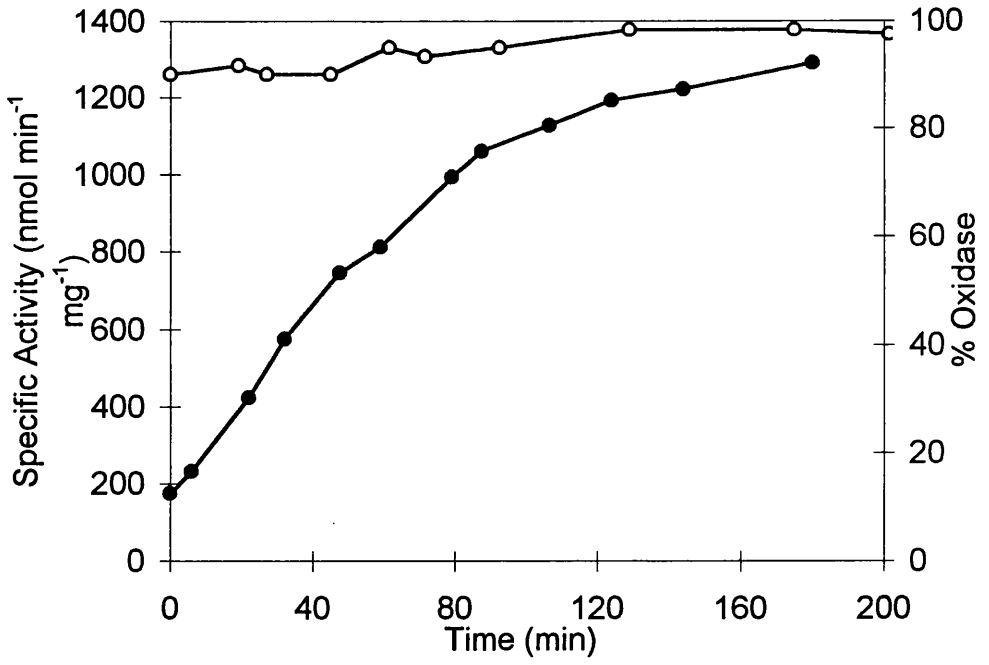


Fig. 8.4 Conversion of BMXDH to BMXO by incubation in air-equilibrated buffer at 37°C. BMXO was incubated in air equilibrated buffer at 37°C as described in Section 8.2.2. Enzyme was assayed over the incubation and the specific xanthine oxidase activity and percentage of oxidase activity calculated. (O) total xanthine oxidase activity, (●) percentage oxidase activity.

8.3.3 XDH to XO Conversion using DTDP

During the incubation of HMXOR in the presence of 1 mM DTDP, the percent oxidase activity increased from 11% (± 2%) to 64% (± 2%), which was not further increased by prolonged incubation or addition of more DTDP. Untreated HMXO (75% oxidase) was incubated with DTDP as

described above. However, the percentage oxidase of this sample remained unchanged. In similar experiments with BMXOR the percentage oxidase activity increased to 98.0 ± 2.0%. All these conversions were shown to be almost completely reversible to the original dehydrogenase activity by incubation with DTT.

8.3.4 Calculation of free sulphydryl groups

The method for assaying the free sulphydryl groups on xanthine oxidase is based on the method of Saito (1987). 4,4'-DTDP reacts with a sulphydryl group to form 4-thiopyridinyl-cysteine and 4-thiopyridone (Fig. 8.6, scheme 1). The 4-thiopyridinyl-cysteine can then react with an adjacent sulphydryl group to form a disulphide bond and 4-thiopyridone (Fig. 8.6, scheme 2). In this way all available sulphydryl groups react producing an equimolar amount of 4-thiopyridone, which can be measured as described in Section 8.2.4.

The number of free sulphydryl groups involved in the conversion was examined with samples of HMXDH (11% ± 2.0% oxidase) and HMXO (75% ± 2% oxidase). The number of free sulphydryl groups in oxidase and dehydrogenase samples and the difference between them was calculated (Table 8.1) to provide an estimation of the number involved in the conversion.

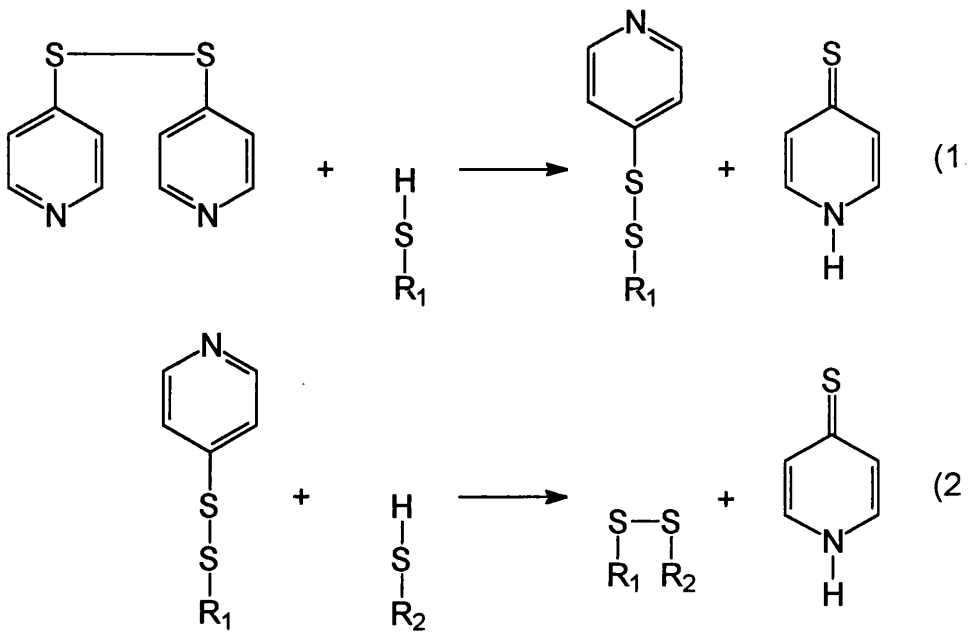


Fig. 8.5. The reaction of DTDP with vicinal thiols. 1) DTDP reacting with one vicinal thiol to produce 4-thiopyridinyl thiol and 4-thiopyridone. 2) 4-thiopyridinyl thiol reacting with another local vicinal thiol to produce 4-thiopyridone and a disulphide bond.

	4-Thiopyridone (mol/mol HMXOR)
HMXDH (10% Oxidase)	13.6 ± 0.8 (n=2)
HMXO (75% Oxidase)	7.6 ± 1.0 (n=2)
Difference	6.0

Table 8.1 Reactivity of HMXO and HMXDH with DTDP. HMXO and HMXDH were reacted with excess DTDP and the formation of 4-thiopyridone was monitored as described in Section 8.2.4. 4-thiopyridone produced is expressed per mole of HMXOR.

8.3.5 XDH to XO Conversions Using Oxidised Glutathione

After incubation with oxidised glutathione, BMXOR was found to have 96% oxidase activity, whereas HMXOR was found to have 45% oxidase activity. Addition of protein disulphide isomerase to HMXOR did not significantly effect the percentage oxidase activity.

8.3.6 Heparin Affinity of HMXDH and HMXO

HMXOR samples (1 mg): unproteolysed HMXO (71% oxidase) prepared by exclusion of DTT from the preparation procedure, unproteolysed HMXDH (8% oxidase) and trypsinised HMXO after incubation with DTT (88% oxidase) were run on FPLC using a heparin column as described in Section 8.2.6. The elution profiles are shown in Fig. 8.6. The peaks of eluted enzyme in molar NaCl were at 0.35, 0.275 and 0.267 for the unproteolysed HMXO, unproteolysed HMXDH and DTT treated proteolysed HMXO respectively.

This experiment indicated a means of separation of the oxidase from the dehydrogenase form of HMXOR. A sample of HMXOR (60 % oxidase) prepared in the presence of DTT, but stored and frozen in its absence, containing a mixture of XO and XDH was applied to the column. The sample eluted as a main peak and a considerable shoulder, at 0.25 and 0.32 M NaCl, shown in Fig. 8.7. These corresponded to the

dehydrogenase and unproteolysed oxidase forms respectively, however the percentages of oxidase forms in both the peak and the shoulder were not significantly different (61% and 62% oxidase respectively).

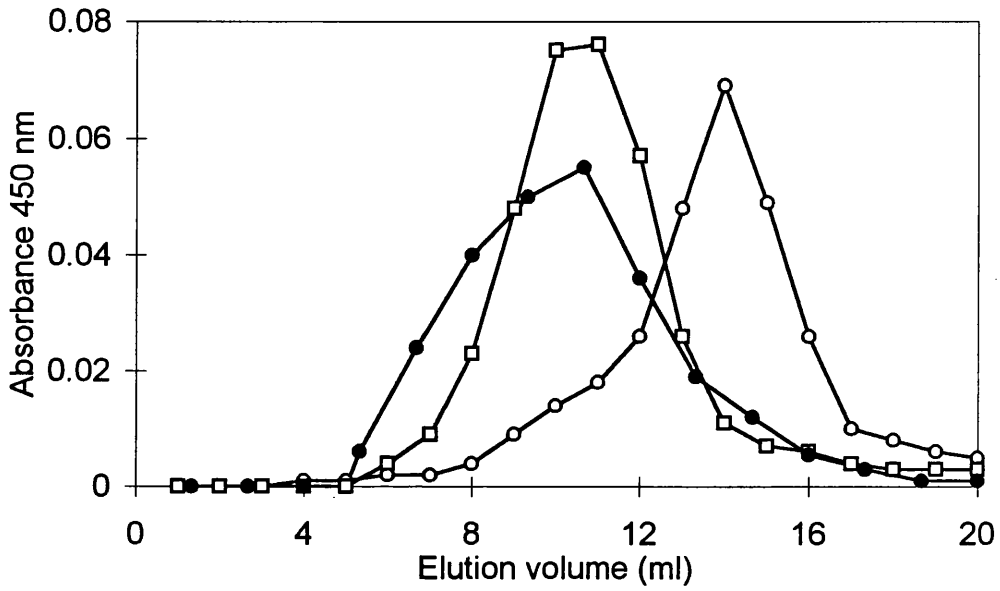


Fig. 8.6. Affinity of HMXOR forms for heparin. HMXOR samples were applied to a heparin agarose column and eluted in a linear gradient of NaCl as described in Section 8.2.6. Fractions were collected and the absorbance at 450 nm measured. Samples were HMXDH (□), unproteolysed HMXO (O) and DTT reduced proteolysed HMXO (●).

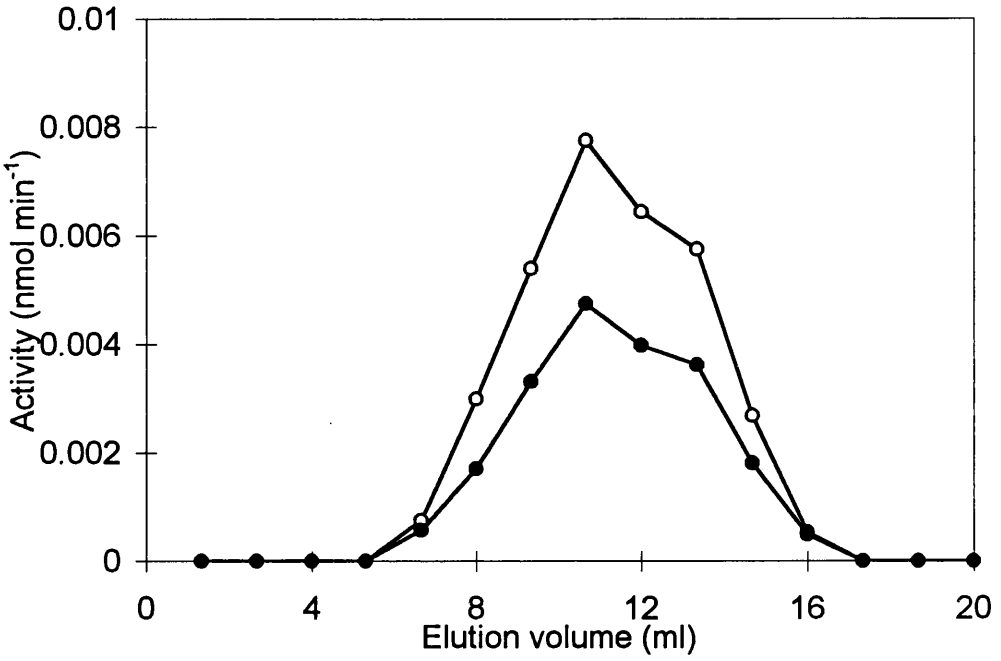


Fig. 8.7. Elution profile of HMXOR (60% oxidase) from heparin agarose. HMXOR was applied to a heparin agarose column and eluted in a linear gradient of NaCl as described in Section 8.2.6. Fractions were assayed for xanthine oxidase activity in the presence (O) and in the absence (●) of NAD.

8.4 Discussion

The XO to XDH conversion of BMXOR with DTT proceeded similarly to those previously reported for the bovine milk and rat liver enzyme (Nakamura & Yamakazi, 1982; Saito, 1987). The XDH to XO conversions of BMXOR with molecular oxygen and DTDP were also similar to those previously reported (Nakamura & Yamakazi, 1982; Waud & Rajagopalan, 1976 b; Saito, 1987; Hunt & Massey, 1992; Stirpe & Della-Corte, 1969). The XO to XDH conversion of HMXOR using DTT was found to be very similar to that of BMXOR. However, the XDH to XO conversions of HMXOR under identical conditions proceeded very slowly and did not reach the very high percentage oxidase activity of BMXOR. A similar finding was observed when HMXOR was converted to the oxidase form in the presence of oxidised glutathione. In contrast to this, proteolysis of HMXOR with trypsin (see Section 8.3.6) was found to yield very largely HMXO.

Saito (1987) reports that chemical modification of sulphydryl groups with reagents such as N-ethylmaleimide inhibits full thiol induced conversion to the oxidase enzyme. Therefore it was considered that in our HMXOR preparations some molecules may have modified sulphydryl groups, possibly as a result of the preparation procedure, which interferes with disulphide bond formation. However, this possibility was dismissed as HMXDH reacted with DTDP to yield 13.6 moles of thiopyridone per mole

of subunit, which matched previous findings in which complete conversion occurred (Waud & Rajagopalan, 1976 b). HMXO (75 % oxidase form), was shown to yield 7.6 moles of thiopyridone per mole of subunit, indicating that 75% oxidase enzyme has on average 6 moles of oxidised sulphydryl groups relating to three disulphide bonds per subunit. Extrapolating this to 100% oxidase enzyme would give 8 oxidised disulphides relating to 4 disulphide bonds. This value fits very well with the reported value of bovine milk and rat liver XOR which requires the oxidation of 8 sulphydryl groups per subunit for conversion to the oxidase form (Hunt & Massey, 1992; Waud & Rajagopalan, 1976 b; Saito, 1987).

Waud and Rajagopalan (1976 b) demonstrated that for the conversion of rat liver XDH to XO, the addition of sulphydryl oxidising reagents was necessary. They also showed that the rapid oxidation of sulphydryl groups, of which some are not involved in the conversion, was followed by a slower change in oxidase activity and therefore postulated that, after oxidation, an additional conformational change was required to cause the conversion. Although HMXDH was seen to oxidise fully, using DTDP, the conversion to the oxidase form did not proceed to its full extent. Therefore the required change in conformation for HMXOR may be either too slow to determine or may be disfavoured.

The heparin affinity of HMXO and HMXDH was investigated, revealing a difference in heparin affinity between HMXO and HMXDH, judged by the peak elution in a gradient of NaCl. The peptides which exhibit the heparin binding capacity in HMXOR (Fukushima *et al.*, 1995) have been determined, and relate to two sequences within the molybdenum domain. Although these peptides have not yet been localised in the XOR molecule, I extrapolated the peptides to those corresponding amino acids in aldehyde oxidoreductase (see Appendix, Section 12.2), a molybdenum containing iron-sulphur enzyme with high sequence homology to XOR and a member of the XOR protein family (Thoenes *et al.*, 1994) where the structure is known (Romao *et al.*, 1995). These peptides were seen to be localised on the surface of XOR (Fig. 8.8).

Both heparin binding peptides are basic, providing the positive charges required for binding to a sulphated glycosaminoglycan, and one of these peptides has been proposed to exist in a helical conformation where the basic residues are localised on one face of the helix (Fukushima *et al.*, 1995).

HMXO was found to have a higher affinity for heparin than HMXDH and trypsinised HMXO after incubation with DTT. The heparin binding capacity of HMXOR appears to be decreased by its reduction with DTT, which implies that, as in the XDH to XO conversion, disulphide bonds are involved. The

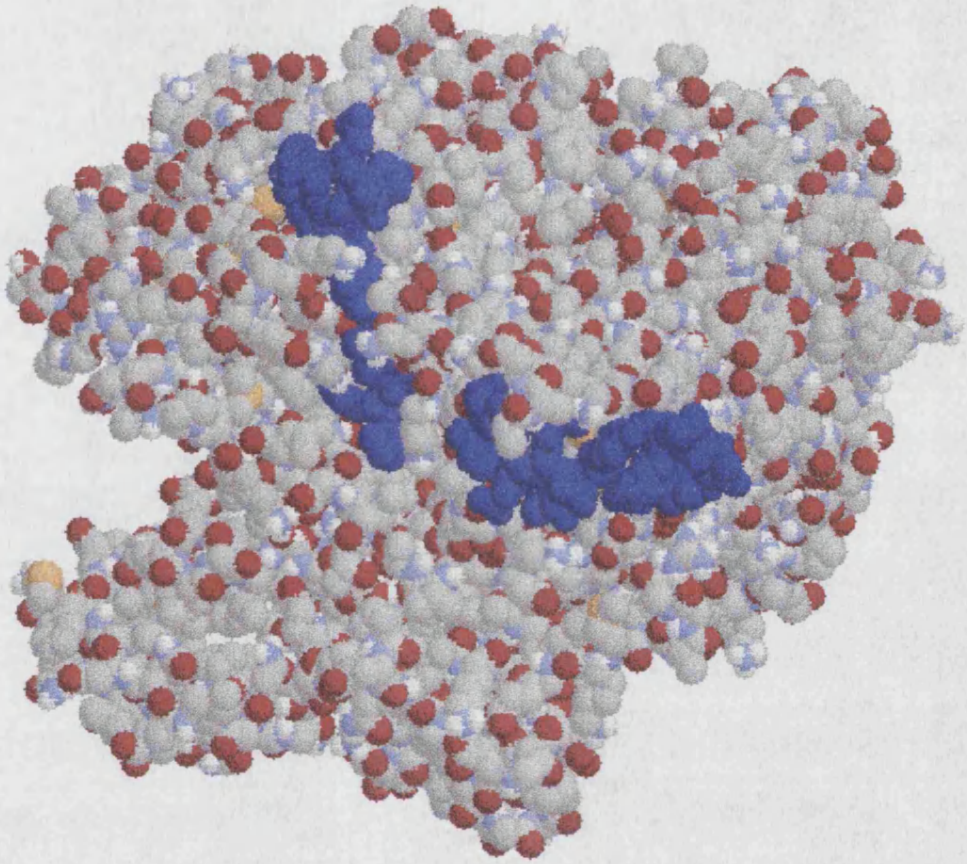


Fig. 8.8. Crystal structure of aldehyde oxidoreductase showing the positions of the heparin affinity peptides (highlighted in blue). The protein sequences of HMXOR and AOR from *Desulfovibrio gigas* were aligned and the amino acids relating to the heparin affinity peptides of HMXOR, amino acids 781 to 795 and 1106 to 1122, were determined. These AOR amino acids, 403 to 417 and 720 to 733, were then highlighted in the crystal structure of AOR using Rasmol modelling software.

formation of these bonds may be responsible for a change in orientation of the heparin binding peptides, therefore altering their heparin affinity. DTT reduced proteolysed HMXO (88% oxidase), which has been shown to have a similar number of free sulphydyl groups as XDH (Waud & Rajagopalan, 1976 b), has a similar elution profile to that of reduced HMXDH, indicating that different factors effect the heparin affinity than those which determine the oxidase activity. In addition, a preparation of HMXOR containing an intermediate amount of XO and XDH was not separated by heparin on the basis of oxidase activity. Intriguingly, although most of the activity was in the oxidase form, most of the protein elutes in the same position as the reduced enzyme. These experiments point to a sulphydryl oxidisable increase in heparin affinity for HMXOR, which is independent of that involved in the XDH to XO conversion and also indicates that the existence of oxidase form does not effect the oxidisable heparin affinity. The presence of the heparin affinity peptides at the surface of the molybdenum domain indicates a disulphide bond formation in this locality, which is different to the disulphide bond or bonds formed during the conversion of XDH to XO. The latter have been thought to be located in or close to the FAD domain. It has been shown that during oxidation of XDH there are 4 disulphide bonds formed, yet not all of these are involved in the D to O conversion (Saito, 1987). Therefore it seems likely that some of those other disulphides are involved in generating the increased heparin affinity.

9 Kinetic Studies

9.1 Introduction

It has been previously reported that purified HMXOR shows very low activity to most reducing substrates, compared to BMXOR (Abadeh *et al.*, 1992). Uniquely, activity toward NADH, which directly reduces FAD rather than molybdenum is similar to that of BMXOR (Sanders *et al.*, 1997). The early data of Abadeh *et al.* were generated with enzyme considerably less pure than that recently available, and those of Sanders *et al.*, while derived from enzyme of high purity were largely confined to NADH oxidase activities. In this chapter, I describe experiments designed to determine kinetic parameters for HMXDH and HMXO and, where possible, to compare them with those for BMXOR. In view of significant variation in the molybdenum site activity between batches of HMXOR, comparisons were in many cases done by using a single large batch of purified enzyme.

9.2 Materials and Methods

9.2.1 Steady-state kinetic assays

Steady-state kinetic studies of xanthine oxidase activity were carried out in air-saturated 50 mM Na-Bicine, pH 8.3, at 25°C. XO and XDH assays were carried out in the absence and presence respectively of 500 μM NAD^+. The rate of oxidation of xanthine to uric acid was measured spectrophotometrically at 295 nm, using an absorption coefficient for uric acid of 9600 M^{-1} cm^{-1} (Avis *et al.*, 1956 a).

NADH oxidase assays were carried out at 25°C in air-saturated 50 mM MES at pH 6.5, 50 mM sodium phosphate at pH 7.4 and 50 mM MOPS at pH 7.5. The rate of oxidation of NADH to NAD^+ was measured spectrophotometrically at 340 nm, by using an absorption coefficient for NADH of 6220 M^{-1} cm^{-1} (Horecker & Kornberg, 1948).

Superoxide production was determined in terms of superoxide-dismutase-inhibitable (30 units) reduction of 25 μM cytochrome c (horse heart), monitored at 550 nm and calculated by using an absorption coefficient for reduced cytochrome C of 21000 M^{-1} cm^{-1} (Massey, 1959).

For substrates that cannot be monitored directly, assays were performed according to Krenitsky et al. (1986). Steady-state kinetic assays were performed at 37°C in 50 mM potassium phosphate, pH 6.8, containing 0.3 mM EDTA, 0.3 mM phenazine ethosulphate (PES), and 0.08 mM cytochrome C (horse heart). The reduction of cytochrome C was monitored at 550 nm and calculated by using an absorption coefficient for reduced cytochrome C of 21000 M^{-1} cm^{-1} (Massey, 1959).

Data were analysed by using ENZPAC software which fits data to the Michaelis-Menten equation and calculates kinetic constants using the direct linear method of Eisenthal & Cornish-Bowden (1974). The program calculates values of K_m and V_{max} with errors expressed as 68% confidence limits.

9.2.2 pH Profiles

pH Profiles were determined by measuring the rate of oxidation at 25°C of 100 μM xanthine or NADH in the presence of 500 μM NAD^+ (Section 3.2.3), in air-saturated 50 mM MES at pH 6 and pH 6.5, 50 mM MOPS at pH 7 and pH 7.5, 50 mM Bicine at pH 8, pH 8.3 and pH 8.5 and 50 mM CHES at pH 9.

9.2.3 Quantification of intermediates in purine oxidation to urate

Cleere *et al.* (1975) describe a mathematical procedure for the estimation of hypoxanthine and xanthine intermediates in the oxidation of purine to urate. Isosbestic points were determined from spectra of purine, hypoxanthine, xanthine and urate at equimolar concentrations. Using these isosbestic points, the concentrations of the substrates can be calculated from UV absorption spectra of the reaction of purine with HMXOR using the following equations:

$$P = \frac{F_{xu1}(A_{h2}-A_{xu2})-F_{xu2}(A_{h1}-A_{xu1})+(A_{xu2}A_{h1}-A_{xu1}A_{h2})}{A_{p1}(A_{h2}-A_{xu2})-A_{p2}(A_{h1}-A_{xu1})+(A_{xu2}A_{h1}-A_{xu1}A_{h2})}$$

$$H = \frac{F_{xu1}(A_{p2}-A_{xu2})-F_{xu2}(A_{p1}-A_{xu1})+(A_{xu2}A_{p1}-A_{xu1}A_{p2})}{A_{h1}(A_{p2}-A_{xu2})-A_{h2}(A_{p1}-A_{xu1})+(A_{xu2}A_{p1}-A_{xu1}A_{p2})}$$

P = concentration of purine

H = concentration of hypoxanthine

F = experimentally derived absorbance at the xanthine urate isosbestic points 1 and 2, F_{xu1} and F_{xu2} respectively

A = absorbance of the intermediate from the standard spectra at the xanthine urate isosbestic points 1 and 2, eg. A_{h1} is the absorbance of hypoxanthine and A_{xu1} is the absorbance of xanthine (or urate) at the xanthine urate isosbestic point 1.

In a similar manner, concentrations of the other intermediates were calculated.

9.3 Results

9.3.1 Xanthine dehydrogenase and oxidase activities of HMXOR and BMXOR

Assays were performed over a range of xanthine concentrations from 1 μM to 100 μM. Steady-state kinetic parameters for the oxidation of xanthine by human and bovine XO and XDH are shown in Table 9.1. The percentage oxidase activities of the samples were determined as described in Section 3.2.4.

	HMXDH (9%)	BMXDH (8%)	HMXO (68%)	BMXO (90%)
K_m (μM)	10.1 (9.1-12.7)	4.8 (4.3-5.2)	8.8 (8.1-9.5)	7.3 (6.7-8.0)
V_{max} (nmol min^{-1} mg^{-1})	82.5 (78.5-96.5)	1377 (1353-1400)	35.8 (34.6-36.2)	1359 (1330-1405)

Table 9.1. Kinetic parameters for xanthine oxidation by human and bovine XDH and XO. Assays were carried out in air-saturated 50 mM Na-Bicine, pH 8.3, as described in Section 9.2.1, For XDH assays, 500μM NAD^+ was additionally present. Percentage values in brackets relate to oxidase contents of the enzyme determined as described in Section 3.2.4. 68% Confidence limits for the calculated parameters are also shown in parentheses.

9.3.2 NADH oxidase activities of human XDH and XO

Assays were performed over a range of NADH concentrations from 0.5 μM to 100 μM. Steady-state kinetic parameters for the oxidation of NADH by human XO and XDH are shown in Table 9.2. The percentage oxidase activities of the samples were determined as described in Section 3.2.4.

	HMXDH (9%)	HMXO (68%)
K_m (μM)	1.0 (0.8-1.1)	2.2 (1.8-2.5)
V_{max} (nmol min^{-1} mg^{-1})	124.3 (124.3-125.5)	88.8 (86.2-90.7)

Table 9.2. Kinetic parameters for NADH oxidation by human XDH and XO. Assays were performed in air-saturated 50 mM sodium phosphate, pH 7.4, as described in Section 9.2.1. Percentage values in brackets relate to oxidase contents of the enzyme determined as described in Section 3.2.4. 68% Confidence limits for the calculated parameters are also shown in parentheses.

9.3.3 pH Profiles of total xanthine oxidase and NADH oxidase activities of HMXO and HMXDH

HMXO (60 % oxidase) was assayed for total xanthine oxidase activity in buffers of different pH containing 100 μM xanthine and 500 μM NAD^+ as described in Section 3.2.3. HMXDH (9 % oxidase) was assayed for NADH oxidase activity in buffers of different pH containing 100μM NADH (Section 3.2.5). The results from these assays are shown in Fig. 9.1.

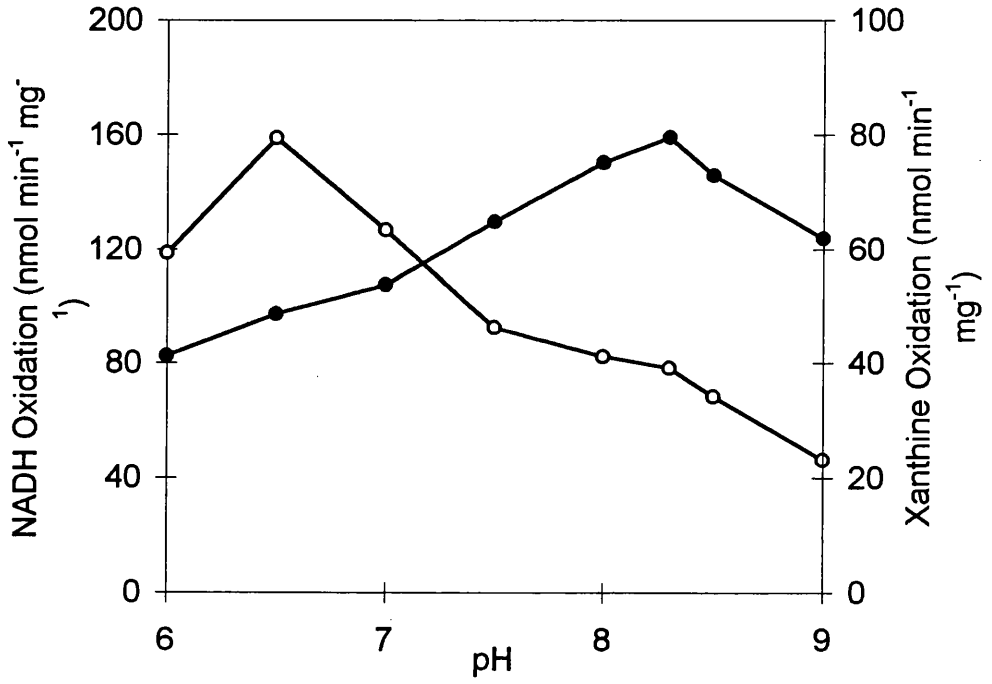


Fig. 9.1. pH-Dependence of total xanthine oxidase and NADH oxidase activities of HMXO and HMXDH (60% and 9% oxidase respectively). Xanthine oxidase (●) and NADH oxidase (O) activities of HMXO and HMXDH were assayed as described in Section 9.2.2 at pH values between 6 and 9.

9.3.4 Comparison of substrate specificities of HMXOR and BMXOR

Assays were performed using the PES cytochrome C assay of Krenitsky *et al.* (1986), as described in Section 9.2.1. Steady-state kinetic parameters for the oxidation of purine, hypoxanthine, xanthine and 2-aminopurine ribonucleoside by HMXOR and BMXOR are shown in Table 9.3.

	HMXO		BMXO	
	K_m (μM)	V_{max} (%X)	K_m (μM)	V_{max} (%X)
Purine	0.52 (0.37-1.23)	0.98 (0.95-1.29)	0.35 (0.25-0.52)	2.45 (2.35-2.57)
Hypoxanthine	7.18 (6.82-7.75)	178 (172-189)	5.25 (4.55-5.53)	217 (214-222)
Xanthine	2.81 (2.73-2.95)	100 (90-102)	2.54 (2.18-2.83)	100 (94-108)
2-APR	356 (313-407)	4.5 (4.25-4.7)	3870 (3360-4000)	7.83 (7.5-8.07)
	HLXO (Krenitsky *et al.*)		BMXO (Krenitsky *et al.*)	
	Km (μM)	Vmax (%X)	Km (μM)	Vmax (%X)
Purine	<1.0	>1.4	0.7	6.3
Hypoxanthine	9.0	220.0	6.0	175.0
Xanthine	7.0	100.0	8.0	100.0
2-APR	890.0	6.3	4000.0	61.0

Table 9.3. Kinetic parameters for the oxidation of purine, hypoxanthine, xanthine and 2-aminopurine ribonucleoside by HMXO and BMXO (9% and 68% oxidase respectively). Substrate-dependent PES cytochrome C assays were performed as described in Section 9.2.1. The values of V_{max} are expressed as a percentage of that for xanthine. The values in brackets relate to 68% confidence limits for the calculated parameters. Also included for comparison are the data of Krenitsky *et al.* (1986) for human liver XO (HLXO) and BMXO. These workers quote average standard errors for their K_m values of ±13% and for their V_{max} values of ±5%.

9.3.5 Quantification of intermediates in the oxidation of purine to urate catalysed by HMXO

UV absorbance spectra of 0.1 mM purine, hypoxanthine, xanthine and uric acid in 50 mM potassium phosphate buffer, pH 7.8, at 25°C, are shown in Fig. 9.2, from which isosbestic points were determined.

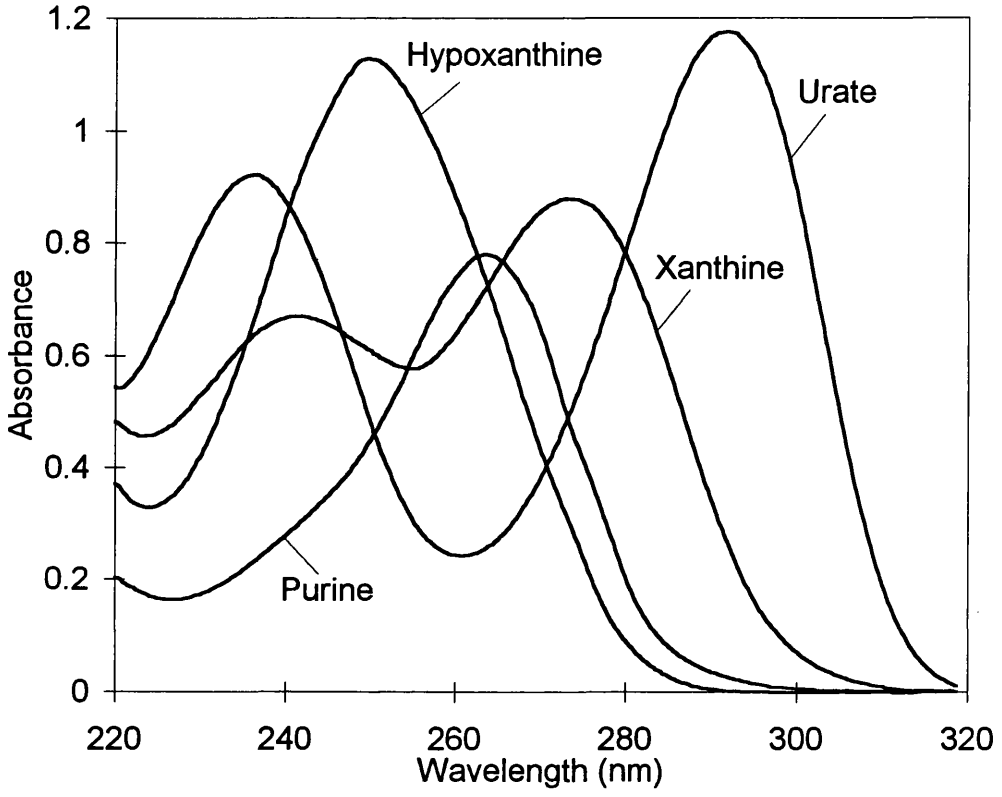


Fig. 9.2. Ultraviolet absorbance spectra of equimolar concentrations of purine, hypoxanthine, xanthine and uric acid. The concentration of each compound was 0.1 mM in 50 mM potassium phosphate buffer, pH 7.8, at 25°C.

HMXO (0.175 mg) was added to 0.1 mM purine in 50 mM potassium phosphate buffer, pH 7.8, and the spectra of the mixture were taken periodically until the purine was nearly exhausted. Selected spectra are shown in Fig. 9.3, and the values at the predetermined isosbestic points were used to calculate the concentrations of the intermediates over time (Fig. 9.4) as described in Section 9.2.3.

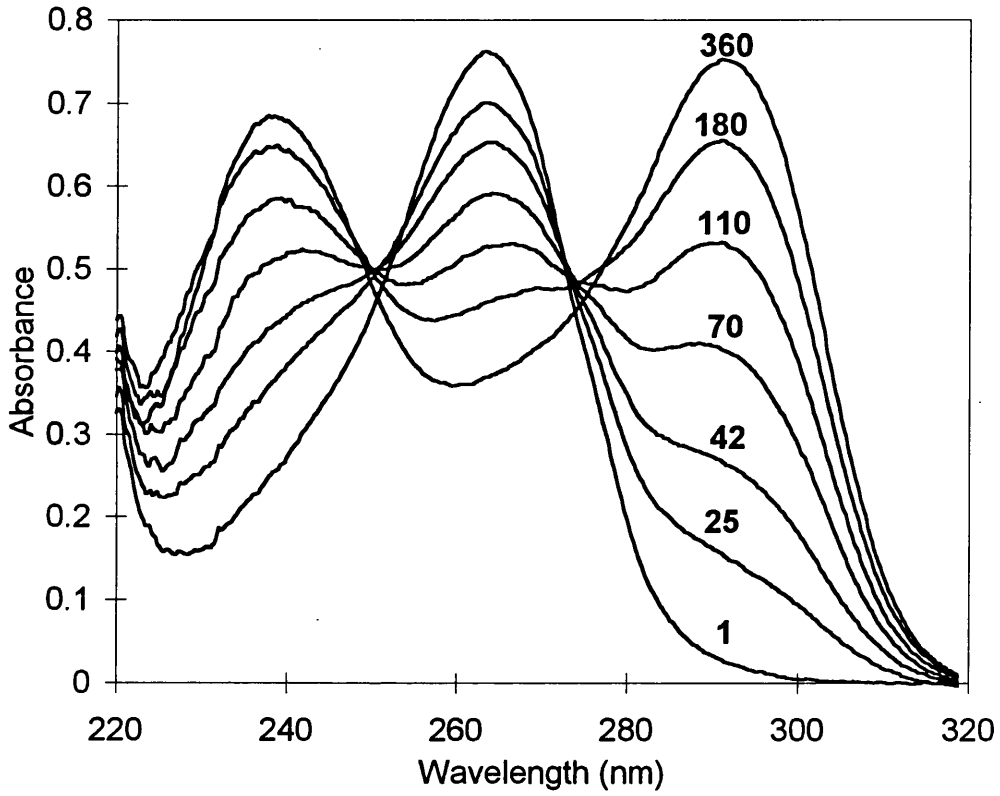


Fig. 9.3. Ultraviolet absorbance spectra of purine oxidation by HMXO. HMXO (68 % oxidase) (1.5 nmol), was added to 0.1 mM purine in 50 mM potassium phosphate, pH 7.8, and incubated at 25°C. Spectra were recorded at the times (min) indicated on the curve.

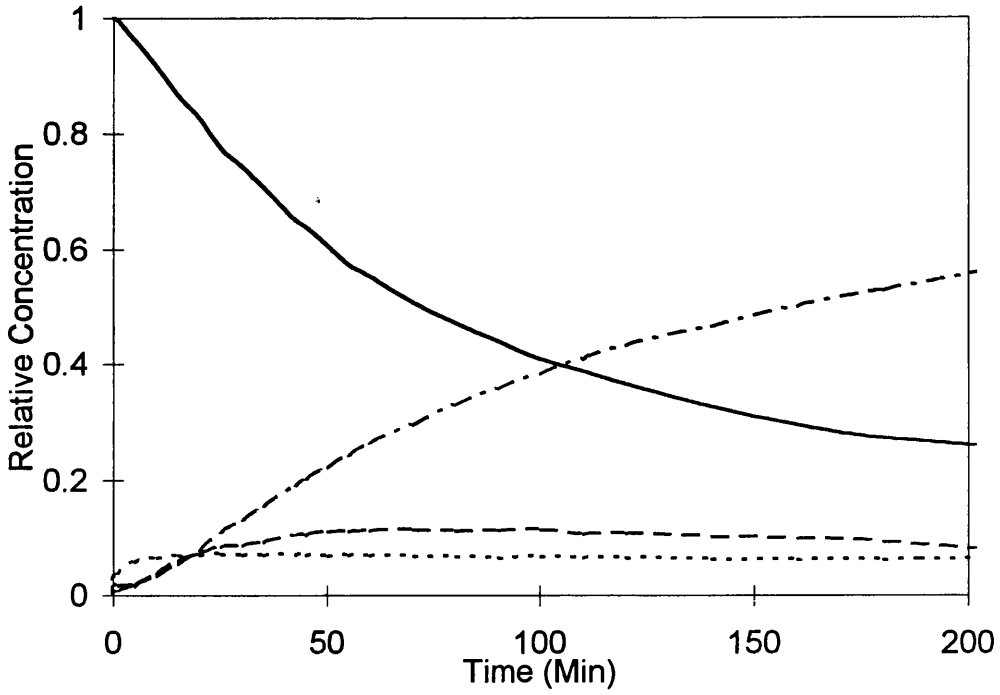


Fig. 9.4. Time course of purine oxidation by HMXO. Concentrations of intermediates in the time-course of purine oxidation by HMXO were calculated as described in Section 9.2.3. The graph shows the utilisation of purine (——), the production of urate (- — -), and the formation of the intermediates hypoxanthine (·····) and xanthine (-----).

9.3.6 pH Profile of NADH oxidase activity and superoxide production by HMXDH

NADH oxidase activity of HMXDH was monitored as a function of pH, in buffers with pH ranging from 6 to 8 containing 500 μM NADH, as described in Section 9.2.2. At all pH values above 6 no measurable non-

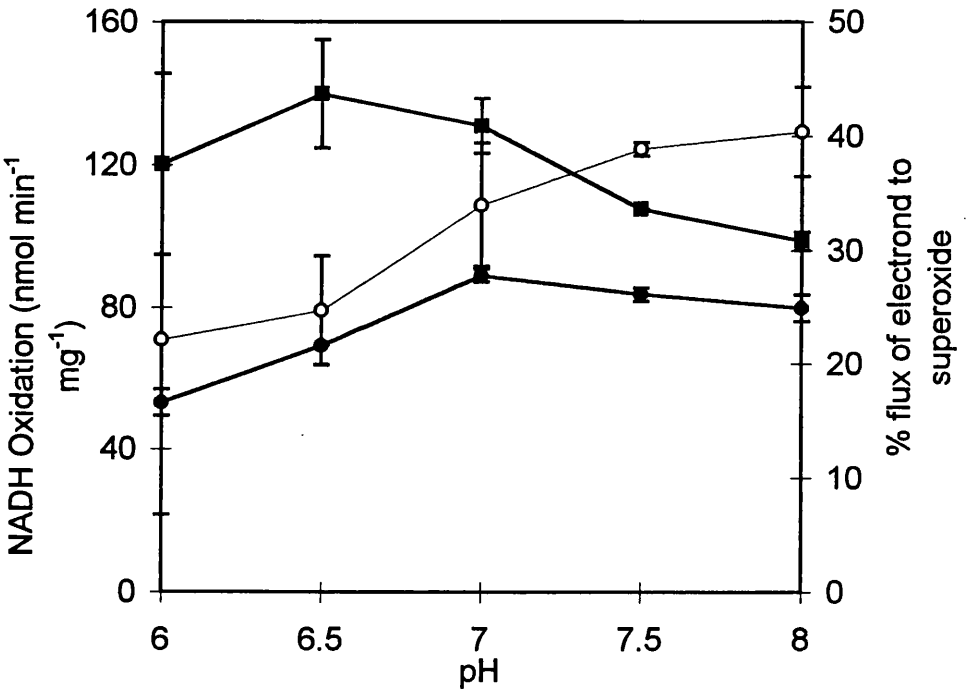


Fig. 9.5. pH-Dependence of NADH oxidase activity and superoxide production by HMXDH. NADH oxidase activity (■) of HMXDH was assayed as described in Section 9.2.2 at pH values between 6 and 8. Superoxide production (●) was also measured as described in Section 9.2.1 and the percentage of electron flux to superoxide (O) was calculated. Error bars relate to standard errors with n=3.

enzymic NADH degradation was observed, however at pH 6 a rate of non-enzymic degradation of 0.2 μM NADH min^{-1} was observed which was subtracted from the rates. Superoxide production from the oxidation of NADH over the range of pH was also monitored as described in Section 9.2.1. From these data the percentage of electron flux to superoxide was calculated and is shown, together with the other data in Fig. 9.5.

9.3.7 NADH oxidase activity of HMXDH at pH 6.5 and pH 7.5

Assays on HMXDH were performed over a range of NADH concentrations from 0.5 μM to 50 μM at pH 6.5 and pH 7.5 to obtain the steady-state kinetic constants for the oxidation of NADH as described in Section 9.2.1. the calculated constants K_m and V_{max} are shown in Table 9.4.

pH	K_m (μM)	V_{max} (nmol/min/mg)
6.5	0.59 (0.52 - 0.65)	146 (143 - 147)
7.5	1.08 (0.99 - 1.20)	123 (119 - 126)

Table 9.4. Kinetic parameters for NADH oxidation by HMXDH at pH 6.5 and pH 7.5. Assays were performed in air-saturated 50 mM MES, pH 6.5, and 50 mM MOPS, pH 7.5, as described in Section 9.2.1. The values in brackets relate to 68% confidence limits for the calculated parameters.

A separate set of experiments was carried out to determine the relative proportions of superoxide and hydrogen peroxide produced. The rates of NADH oxidation and superoxide production were monitored at pH 6.5 and pH 7.5 as described in Section 9.2.1 and are shown in Fig. 9.6 and Fig. 9.7 respectively. From these data the percentage electron flux to superoxide was calculated (Fig. 9.8).

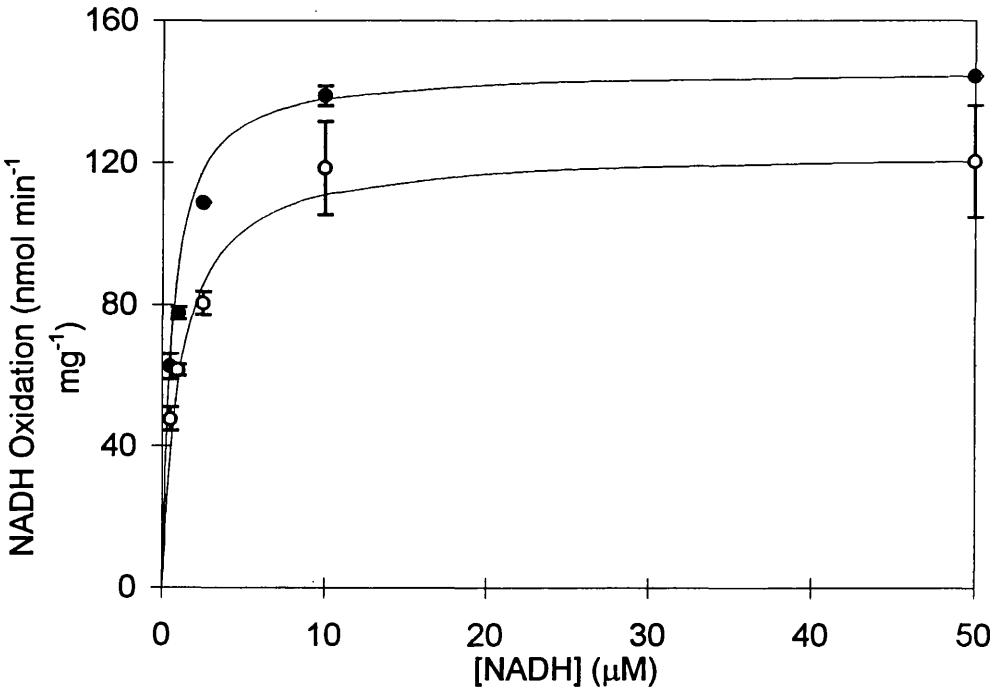


Fig. 9.6. NADH oxidation by HMXDH at pH 7.5 and pH 6.5. NADH oxidation was measured spectrophotometrically as described in Section 9.2.1 in air-saturated 50 mM MOPS, pH 7.5, (O) and 50 mM MES, pH 6.5, (●). The data were fitted to Michaelis-Menton curves using the figures in Table 9.4. Error bars relate to standard errors with n=3.

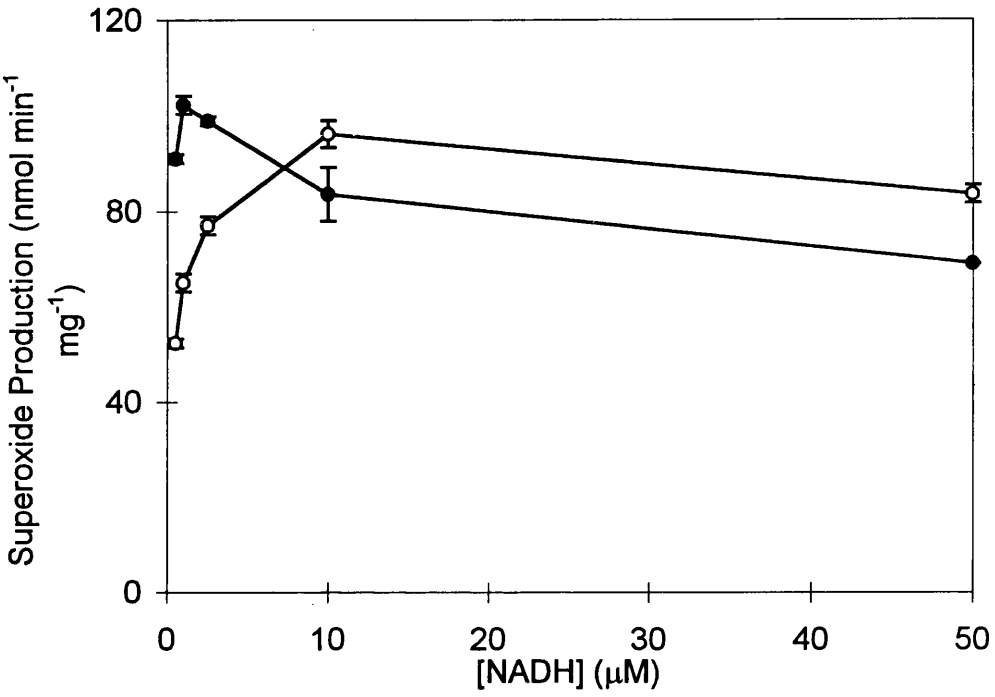


Fig. 9.7. Superoxide production by HMXDH at pH 7.5 and pH 6.5. The rates of superoxide were determined as described in Section 9.2.1 in air-saturated 50 mM MOPS, pH 7.5, (O) and 50 mM MES, pH 6.5, (●). Error bars relate to standard errors with n=3.

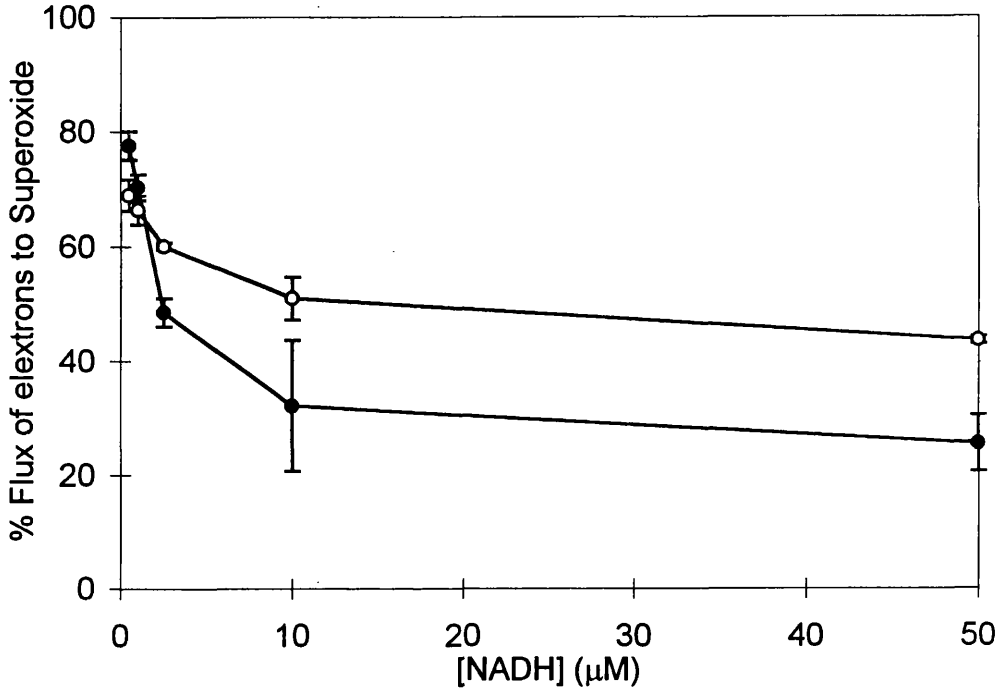


Fig. 9.8. The % electron flux to superoxide from the oxidation of NADH by HMXDH at pH 7.5 and pH 6.5. The percentage electron flux to superoxide was calculated from the data in Fig. 9.6 and Fig. 9.7 in air-saturated 50 mM MOPS, pH 7.5, (O) and 50 mM MES, pH 6.5, (●). Error bars relate to standard errors with n=3.

9.3.8 Effect of NAD^+ on superoxide generation catalysed by HMXDH in the presence of xanthine

Oxidation of xanthine (100μM) catalysed by HMXDH with and without 500 μM NAD^+ in air-saturated 50 mM sodium phosphate, pH 7.4, and 50 mM MES, pH 6.5, was monitored (Section 9.2.1). NADH production was also measured spectrophotometrically by monitoring the increase in absorbance at 340 nm, using an absorption coefficient for NADH of 6220 M^{-1} cm^{-1} (Horecker & Kornberg, 1948). The results are shown in (Table 9.5).

pH	% Oxidase Absence of NAD^+	% Dehydrogenase Presence of NAD^+	Remaining % Xanthine Oxidation
7.4	12.9	70.5	16.6
6.5	13.6	43.7	42.7

Table 9.5. The effect of NAD^+ on % oxidase and dehydrogenase activities of HMXO at pH 7.4 and pH 6.5. The percentage oxidase activity was measured as described in Section 3.2.4 in air-saturated 50 mM sodium phosphate pH 7.4 and 50 mM MES pH 6.5 at 25°C. The percentage dehydrogenase activity in the presence of 500μM NAD^+ was calculated as described in section 9.3.8. The remaining xanthine oxidase activity after subtraction of the oxidase and dehydrogenase activities is also shown.

Calculation of the electron flux to oxygen by monitoring xanthine oxidation in the absence of NAD^+ (Section 3.2.4), accounts for only 13% of that in the presence of 500 μM NAD^+ at pH 7.4 and pH 6.5. This demonstrates that at least 87% of the electrons from xanthine should reduce NAD^+ in the presence of 500 μM NAD^+. However, the production of NADH was monitored and accounts for only 71% and 44% of the xanthine oxidised at pH 7.4 and 6.5 respectively, a difference of 29% and 56% at the respective pH. These conflicting results suggest that in the presence of NAD^+, the flux of electrons to oxygen is actually increased from 13% to 29% or to 56%, at pH 7.5 and 6.5 respectively.

HMXDH (8% oxidase) was assayed in air-saturated 50 mM MES, pH 6.5, containing 100 μM xanthine, and concentrations of NAD^+ from 0 to 500 μM. Xanthine oxidation was monitored at 295 nm and NAD^+ reduction was monitored at 340 nm as described above and the rates of xanthine oxidation were calculated (Fig. 9.9). The data show that addition of 2 to 500 μM NAD^+ increases the rate of urate production by HMXDH. NADH production was not observed at any NAD^+ concentration except at 500μM NAD^+. At lower NAD^+ concentrations, as NADH production was not observed, the flux of electrons to oxygen must account for the total amount of xanthine utilised. At 500μM NAD^+ significant NADH production was seen which initially accounted for approximately 44% of the xanthine oxidised, as described above. The data from the time course of this assay

over 20 min of turnover were plotted (Fig. 9.10) which show that the rate of NADH production was not sustained during the oxidation of xanthine, and after 10 min the NADH that was initially produced is oxidised.

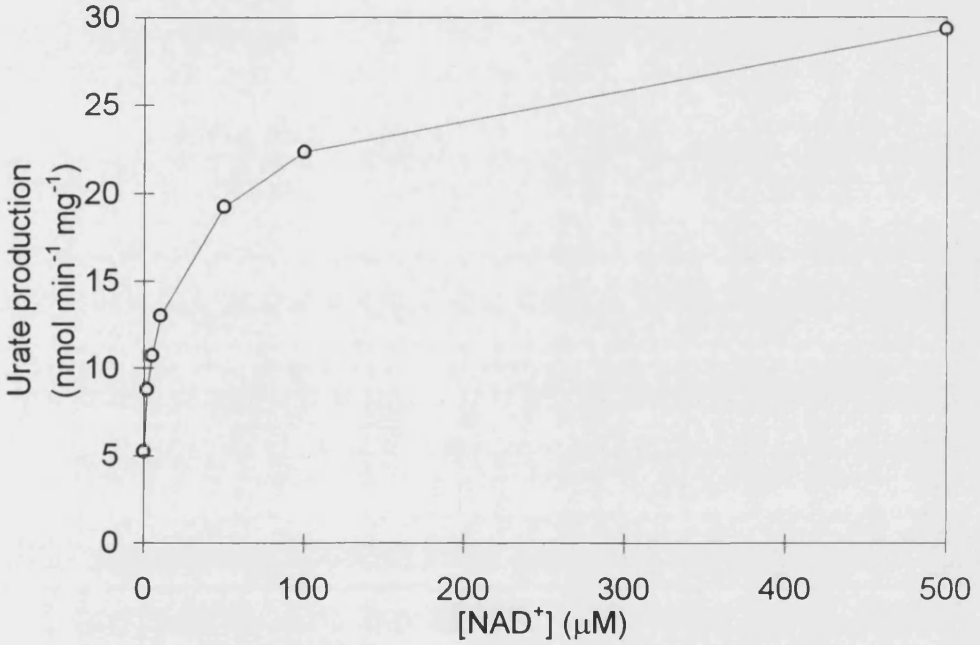


Fig. 9.9. The rate of urate production of HMXDH in the presence of varying concentrations of NAD^+. HMXDH was assayed at 25°C in air-saturated 50 mM MES, pH 6.5, containing 100 μM xanthine and 0 to 500μM NAD^+. Urate production was monitored at 295 nm and was calculated, as described in Section 9.2.1.

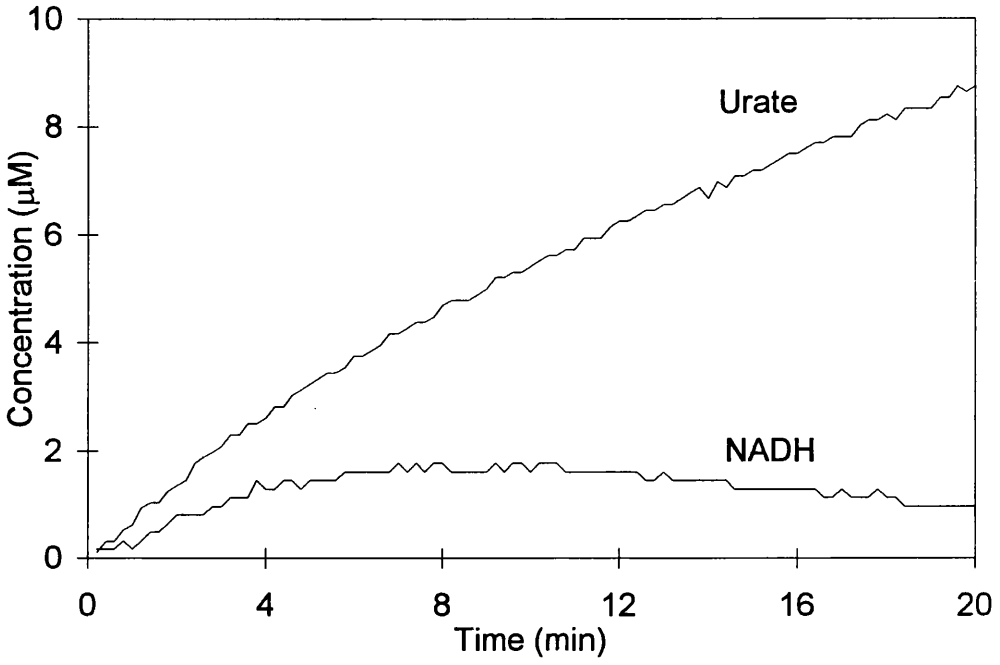


Fig. 9.10. Urate and NADH production during turnover with HMXDH. HMXDH was assayed at 25°C in air-saturated 50 mM MES, pH 6.5, containing 100 μM xanthine and 500μM NAD^+. Urate production was monitored at 295 nm and NADH production was monitored at 340 nm and the amounts of xanthine oxidised and NADH produced were calculated as described in Section 9.2.1.

The fate of the electrons reducing oxygen was considered by monitoring the production of superoxide in the above system. HMXDH was assayed in air-saturated 50 mM MES, pH 6.5, containing 100 μM xanthine, 25 μM cytochrome C and concentrations of NAD^+ from 0 to 500 μM. Cytochrome C reduction was monitored at 550 nm as described in Section 9.2.1 and the rates of superoxide production and percentage flux of electrons to superoxide were calculated (Fig. 9.11 & Fig 9.12).

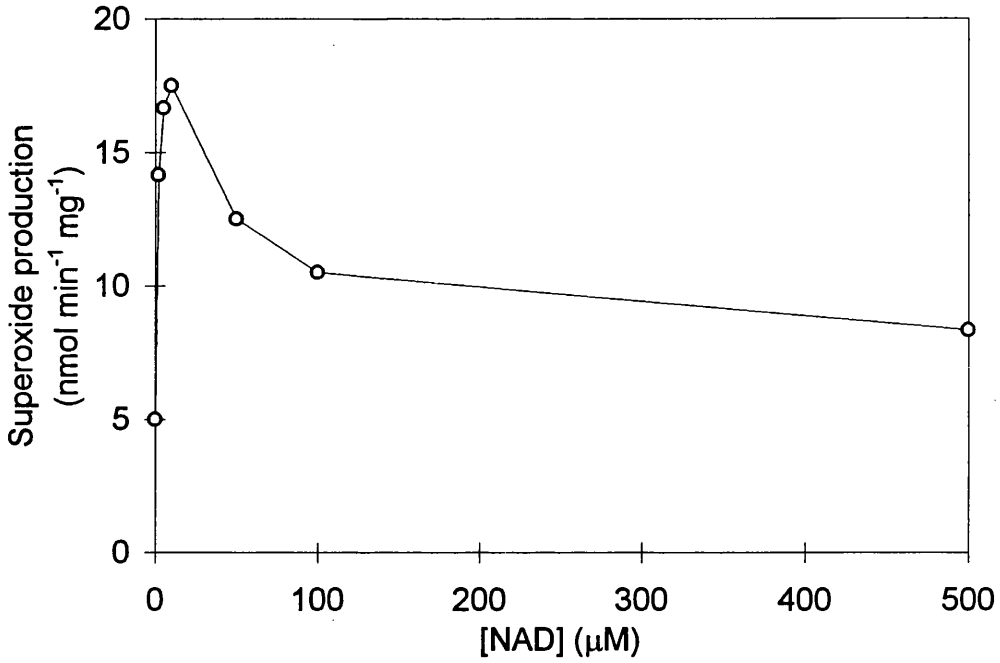


Fig. 9.11. The effect of NAD^+ on superoxide production of HMXDH in the presence of xanthine. HMXDH was assayed at 25°C in air-saturated 50 mM MES, pH 6.5, containing 50 μM xanthine, 25μM cytochrome C and 0 to 200μM NAD^+. Cytochrome C was monitored at 550 nm and the rate of superoxide production was calculated, as described in Section 9.2.1.

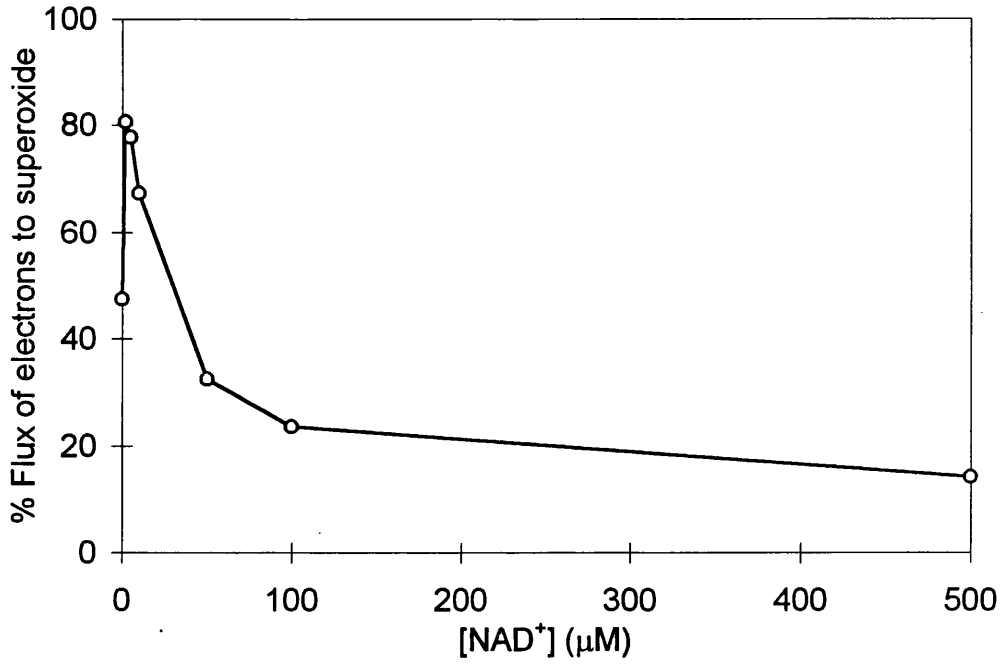


Figure 9.12. The % electron flux to superoxide from the oxidation of Xanthine in the presence of varying concentrations of NAD^+. The percentage electron flux to superoxide was calculated from the data in Fig. 9.9 and Fig 9.11 as described in Section 9.3.8.

The data show that addition of 2 to 500 μM NAD^+ increases the rate of xanthine oxidation, yet only at the highest NAD^+ concentration was NADH production detected. Superoxide production was greatest at 10 μM NAD^+. The % flux of electrons to superoxide was highest at the lowest concentration of NAD^+ (2 μM) where it accounted for 80 % of all xanthine oxidised.

The largest rate of superoxide production by HMXDH from xanthine (100 μM) was in the presence of 10μM NAD^+. This concentration was studied at the more physiologically relevant pH of 7.5. HMXDH was assayed in 50 mM MOPS, pH 7.5, containing 100 μM xanthine, 25 μM cytochrome C, with and without 10 μM NAD^+. Urate production and cytochrome C reduction was monitored and the rates of xanthine oxidation and superoxide production were calculated (Section 9.2.1). From this the percent flux of electrons to superoxide was determined (Table 9.6). Here we see that the consumption of xanthine by HMXDH increases by approximately 50% in the presence of 10 mM NAD^+, the production of superoxide increases by 180% and the percent flux of electrons to superoxide increased to 37% at pH 7.5.

NAD^+ (μM)	Xanthine Oxidation (nmol/min/mg)	Superoxide production (nmol/min/mg)	electron flux to O_2^- (%)
0	18.3 ± 0.0	7.1 ± 0.7	19.6 ± 4.7
10	26.6 ± 0.8	19.8 ± 0.6	37.3 ± 3.0

Table 9.6. The effect of NAD^+ on the flux of electrons from xanthine to superoxide at pH 7.5. HMXDH was assayed for xanthine oxidation and superoxide production in 50 mM MOPS, pH 7.5 at 25°C in the absence and presence of 10 μM NAD^+ as described in Section 9.2.1. The percentage flux of electrons, from the oxidation of xanthine, producing superoxide was calculated.

9.4 Discussion

The Michaelis constants for the oxidation of xanthine by BMXO (Table 9.1) are in agreement with those of Hille and Nishino (1995), the K_m for xanthine oxidation by BMXDH was 10 fold higher than that determined previously (Hunt & Massey, 1992) but these workers used NADH production to assay activity, an assay that for reasons of comparison with HMXDH, was not chosen as it appears flawed for low molybdenum enzymes. The V_{max} values for xanthine oxidation by HMXOR were very much lower than those of BMXOR under identical conditions (Table 9.1). HMXO and HMXDH showed similar K_m values but the V_{max} of the oxidase form is significantly lower.

The NADH oxidase activities of HMXDH and HMXO (Table 9.2) were found to have similar Michaelis constants to those published (Sanders *et al.*, 1997). The oxidase form has a lower V_{max} than that of the dehydrogenase and the K_m of the oxidase form is double that of the dehydrogenase. The specific activities of NADH oxidation for HMXDH and BMXDH were found to be identical after taking into account the differences in molar extinction coefficients (Table 7.1).

The pH profiles of xanthine and NADH oxidation by HMXO (Fig 9.1) are very similar to those published for BMXO (Briley & Eisenthal, 1974; Bray *et al.*, 1996) showing pH maxima at 8.3 and 6.5 for xanthine and NADH

oxidation respectively. It is worth noting that human milk has a pH of 6.5, optimal for NADH oxidation.

In 1986, Krenitsky *et al.* purified XOR in proteolysed oxidase form (HLXO) from post-mortem human liver. Their HLXO showed rates of xanthine oxidase activity that were very similar to those of BMXO, in marked contrast to our HMXO which has very low xanthine oxidase activity. In order to compare substrate specificities of HMXO with those of HLXO and BMXO, K_m and V_{max} values for purine, hypoxanthine, xanthine and 2-aminopurine ribonucleoside (2-APR) were determined. The latter reducing substrate was particularly chosen because the K_m values for HLXO and BMXO had been shown to differ greatly. (Krenitsky *et al.*, 1986; Table 9.3). Table 9.3 shows that kinetic parameters for my BMXO preparation are broadly similar to those of Krenitsky *et al.* apart from the relative (to xanthine) V_{max} for 2-APR, which was very much lower in my preparation. K_m and relative V_{max} values of HMXO were essentially similar to those of my (and Krenitsky's) BMXO with the notable exception of the K_m for 2-APR which was markedly lower in the case of HMXO, being much closer to that of HLXO.

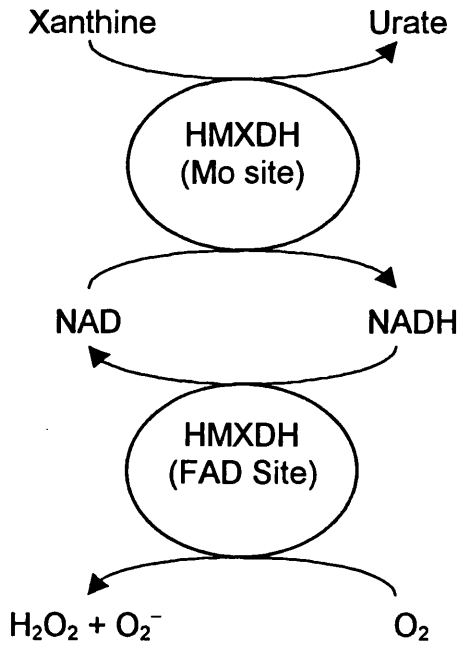
Virtually all forms of XOR oxidise purine to urate via hypoxanthine and xanthine, with the exception of the xanthine oxidase of *Clostridium cylindrosporum*, in which 6,8-dihydroxypurine replaces xanthine

(Bradshaw & Barker, 1960). Cleere *et al.* (1975) describe a mathematical method for the estimation of intermediates from UV absorption spectra of the reaction using isosbestic points. The oxidation of purine to urate by HMXOR proceeded via hypoxanthine and xanthine respectively, as it does for all mammalian enzymes studied (Cleere *et al.*, 1975). The intermediates were found to be hypoxanthine and xanthine which were only accumulated in relatively small amounts compared to purine and urate. The accumulation of intermediates fits with the hypothesis of Jezewska (1973), according to which the intermediates dissociate from the enzyme and, once formed, must compete with other substrates.

As discussed previously, human milk was found to have a pH of 6.5, the pH at which the rate of NADH oxidation by HMXDH is greatest (Fig. 9.1). There is no data on the presence of significant amounts of NAD^+ or NADH in milk. However as the MFGM is derived from the membrane of the milk secreting epithelial cell, the inner side is considered to be cytosolic and therefore will contain both, although the overall concentration in milk may be below the level of detection. In view of this, if NADH oxidase-derived ROS are to have a role in human milk, then it might be expected that, at pH 6.5, HMXOR can produce ROS as has been shown at higher pH (Sanders *et al.*, 1997). The pH profile of NADH oxidation, superoxide production and % flux of electrons to superoxide (Fig. 9.5) shows that, at pH 6.5, while the rate of NADH oxidation is higher, the amount and % flux of electrons to superoxide is actually less than at higher pH, in the

presence of 100 μM NADH. This may be explained in terms of the scheme for NADH oxidation proposed by Sanders *et al.* (1997). HMXDH, which has a lower K_m and a higher V_{max} at pH 6.5, will generally be more reduced in the presence of higher NADH concentrations than at higher pH. These conditions will favour 2 electron transfer to oxygen, generating hydrogen peroxide rather than superoxide as indicated in Fig. 9.7 and Fig. 9.8. Conversely, at very low concentrations of NADH, the percentage flux of electrons to superoxide is much higher and more superoxide is produced. This difference presumably reflects the dramatic reduction of FAD/FADH$^{\bullet}$ midpoint potential at lower pH. At pH 7.5, the midpoint potentials for Fe-S I, Fe-S II, FAD/FADH$^{\bullet}$ and FADH$^{\bullet}$/$FADH_2$ are approximately -306, -208, -274 and -222 mV respectively, whereas at pH 6.5 the same potentials are reduced to -286, -188, -217 and -202 mV (Porass & Palmer, 1982). At both pH values a two electron reduced enzyme would almost certainly have Fe-S II reduced, and the midpoint potentials would favour the second electron to reduce Fe-S I rather than FAD to FADH$^{\bullet}$. At pH 7.4 the differences between the midpoint potentials of Fe-S I and FAD/FADH$^{\bullet}$ is 66 mV whereas at pH 6.5 this difference is reduced to 29 mV. Therefore at pH 6.5 there would be proportionately more FADH$^{\bullet}$ containing enzyme than at pH 7.5, capable of reducing oxygen to superoxide, and therefore proportionately less FAD containing enzyme which is able to be further reduced by NADH and will therefore yield hydrogen peroxide.

The low specific activity of HMXOR toward xanthine and other conventional reducing substrates results from the presence of large amounts of molybdenum-inactive enzyme (Chapter 9). In fact, this activity is of the same order as the specific activity of NADH oxidation, and, indeed, at pH 6.5, the rate of NADH oxidation is actually greater than that of xanthine oxidation in all samples studied. A workable hypothesis for these observations is that the NADH produced from the oxidation of xanthine by HMXDH could be reoxidised by molybdenum-inactive enzyme at the unaffected FAD site, as shown in Scheme 9.1.



Scheme 9.1. The oxidation of xanthine by HMXDH yields ROS in the presence of NAD^+ or NADH.

Investigations into the fate of xanthine-derived electrons, showed that, in the absence of NAD^+, the rate of xanthine oxidation was 13% of that in the presence of NAD^+. From this, given that in the presence of NAD^+ the rate of oxygen reduction should be reduced, it is expected that, at least, the remaining 87% of xanthine-derived electrons, in the presence of NAD^+, would reduce NAD^+, producing NADH. However, NADH production corresponded to only 71% and 44% of the xanthine-derived electrons at pH 7.4 and pH 6.5 respectively (Table 9.5).

At pH 6.5, in the presence of low NAD^+ concentrations no NADH production was observed showing that 100% of xanthine derived electrons are therefore reducing oxygen. These discrepancies could be explained if a large proportion of NADH that is produced is reoxidised according to Scheme 9.1. At the highest NAD^+ concentration, production of NADH was observed which after considerable turnover was seen to be oxidised (Fig 9.10). At higher NAD^+ concentrations, NADH oxidation is inhibited by NAD^+, as described by Sanders *et al.* (1997), allowing the accumulation of NADH. As the xanthine concentration depletes, the rate of production of NADH will fall, and under these conditions the relatively faster rate of NADH oxidation accounts for the loss of NADH.

The electrons that are reducing oxygen could either produce superoxide or hydrogen peroxide. There was an increase in rate of superoxide

production as increasing concentrations of NAD^+ are added to HMXDH in the presence of xanthine, as shown in Fig. 9.11. At low NAD^+ concentrations a large increase in superoxide production is observed whereas at higher NAD^+ concentrations rate of superoxide production decreases. This appears to be analogous to the results with NADH (Fig. 9.7) where at low NAD^+ concentrations a large proportion of superoxide is produced, which decreases as the NAD^+ concentration increases. A similar finding was observed by Nishino *et al.* (1989 a) where addition of NAD^+ or 3-aminopyridine adenine dinucleotide, a non-reducible analogue, dramatically reduced the percent of superoxide produced by XDH in turnover with xanthine. This compound was found to stabilise $FADH^\bullet$ (Schopfer *et al.*, 1988), and these workers proposed that the superoxide produced by reaction of oxygen with $FADH^\bullet$ may be sufficiently hindered in leaving the flavin that it serves as an electron acceptor for a second oxidation reaction.

This finding that NADH is recycled by XDH in turnover with xanthine was not observed by Nishino *et al.* (1989 a.). These workers however used enzyme of very high molybdenum content (≈75%), performed the assay at a much higher pH of 7.8 and used much higher concentrations of NAD^+. A pH of 7.8 would lead to lower rates of NADH reoxidation and the higher amounts of NAD^+ would cause appreciable inhibition of NADH oxidation compared with my system. Most importantly the higher molybdenum centre activity of their preparation would mean that most enzyme

molecules would undergo reduction by xanthine and therefore decrease the amount of enzyme that is able to be reduced by NADH under these conditions.

The proposed scheme (Scheme 9.1) is therefore unique to preparations of enzyme that contain significant amounts of molybdenum-inactive XOR. Although the rate of production of superoxide from this scheme is still lower than that shown by high activity XO, it does allow the production of ROS from xanthine in the presence of low concentrations of NAD^+ by HMXOR in the physiologically relevant dehydrogenase form. It is worth noting that at high concentrations of xanthine, it has been shown that oxygen is converted to superoxide very inefficiently by XOR (Fridovich, 1970; Powell PhD Thesis 1995). This is because the molybdenum active molecules in the oxidase form will become reduced by 6 electrons, favouring 2 electron reduction of oxygen to hydrogen peroxide (Nishino *et al.*, 1989 a, Hille, 1994). The proposed scheme allows the efficient production of superoxide even from high concentrations of xanthine, at low concentrations of NAD^+. In the cell, however, where NAD^+ concentrations are 400-500 μM (Tischler *et al.*, 1977), proportionately more hydrogen peroxide will be produced.

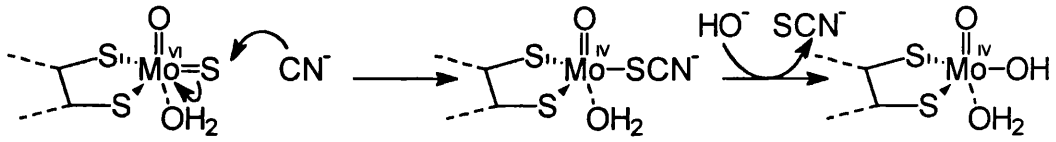
10 Molybdenum Centre Deficiencies

10.1 Introduction

In many respects HMXOR is similar to BMXOR and enzyme from other sources, with the exception of the very low specific activity toward xanthine and other purine substrates. NADH oxidase activity has been shown to be comparable with that from other sources (Abadeh *et al.*, 1992; Sanders *et al.*, 1997). Xanthine and all other reducing substrates, apart from NADH, are known to be oxidised at the molybdenum domain (Hille, 1994), whereas NADH acts at the FAD site (Nishino *et al.*, 1989 b; Nakamura 1991). It has been known for many years that purified XOR preparations can contain significant percentages of molybdenum-inactive enzyme (Morell, 1952; Ventom *et al.*, 1988; Gardlik *et al.*, 1987). In the present work, preparations of BMXOR showed activities toward xanthine similar to literature values, whereas those of HMXOR, prepared by essentially identical procedures, were very low, representing only approximately 5 - 10% of the activity of BMXOR (Chapter 9). These observations, coupled with the low specific xanthine oxidase activity observed in human milk, led us to the conclusion that our inactive HMXOR was not simply an artefact of purification and that the molecular basis for its inactivity merited investigation.

It is well established that BMXOR preparations commonly contain demolybdo enzyme, lacking Moco. Accordingly, both colourimetric and mass spectroscopic determinations of molybdenum of the low activity HMXOR and the relatively higher activity BMXOR preparations were undertaken and the possibility of a lack of Moco was also investigated.

Desulpho enzyme, in which the essential Mo=S group is replaced by a catalytically-inactive Mo=O group, is also known to be a natural component of BMXOR preparations (Nishino *et al.*, 1982; Ikegami & Nishino, 1986). Desulpho enzyme can also be generated *in vitro*. Treatment of active XOR with cyanide causes complete inactivation resulting in the essential sulphur being released as thiocyanate (Massey & Edmondson, 1970), the likely mechanism for this is shown in Scheme 10.1 (Fish *et al.*, 1990).



Scheme 10.1. The inactivation of XOR by removal of the catalytically essential molybdenum-sulphur by cyanide.

This process can be reversed by treatment with sulphide, causing reincorporation of sulphur at the molybdenum site (Massey & Edmondson 1970; Nishino *et al.*, 1982). In the present work, the possible presence of

desulpho enzyme in our HMXOR preparations was investigated by attempted resulphuration under reducing conditions with sodium sulphide, methyl viologen and sodium dithionite.

10.2 Materials and Methods

10.2.1 Colourimetric molybdenum determinations

Colourimetric molybdenum determination was a scaled down version of the method of Hart *et al.* (1970). Known amounts of XOR, containing approximately 10 μmoles of molybdenum in 50 - 100 μl, were wet ashed in 10 ml borosilicate glass sample tubes by adding $HClO_4$:H_2SO_4 (2:3 v/v) (50μl) and heating the open tubes to 260 °C over 3 h, after which all samples became colourless. The samples were cooled and 5M HCl (1.5 ml) was added to each tube, which were then, and subsequently after each addition, stoppered and mixed. $FeSO_4$ [$FeSO_4.7H_2O$ (1 g) in H_2O (20 ml) acidified by addition of conc. H_2SO_4 (0.4 ml)] (50 μl) and 33% (w/v) NaI in H_2O (50 μl) were added and, after 10 min, the brown colour was discharged by sequential additions of 2.5% (w/v) Na_2SO_3 in H_2O (10 μl). Then 10% (w/v) thiourea in H_20 (200 μl), 43% (w/v) L(+)tartaric acid in H_20 (50 μl) and dithiol reagent [0.2% (w/v) 3,4-dimercaptotoluene in 1% (w/v) NaOH with 1.4% (v/v) mercaptoacetic acid added] (200 μl) were added and the tubes were thoroughly mixed by inversion 100 times and left for

30 min. Amyl acetate (200 μl) was added and again the samples were thoroughly mixed by 100 inversions and left until the amyl acetate had separated. The amyl acetate was removed and centrifuged to separate any remaining aqueous layer and the spectra were recorded.

Molybdenum contents were calculated by using the difference in absorbance between 680 and 540 nm. Standards of $(NH_4)Mo_7O_{24}.4H_2O$ and MoO_3 were carried through the process to provide an extinction coefficient and the molybdenum contents of blanks and standards were calculated by using this.

10.2.2 Mass spectroscopy molybdenum estimations

Mass spectroscopy was kindly carried out by Mr P. Turner of Micromass Ltd. Manchester. Samples of XOR, MoO_3 standard and blanks were wet-ashed in conc. $HClO_4/H_2SO_4$ (70:30) by heating in a block to 260°C for 3 h. The samples were then diluted (1 - 10 ppb Mo) in 5% nitric and Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) was performed on the samples. The results were calculated against the standard peak areas for each of the molybdenum isotopes with molecular masses of 92, 94, 95, 96 and 100.

10.2.3 Molybdenum cofactor analyses

The method for the extraction and quantification of molybdopterin is based on the method of Frunzke (1993), where liberated cofactor is modified by carboxamidomethylation by reaction with iodoacetamide to yield di-carboxamidomethyl molybdenum cofactor (di-cam Moco) and quantified by HPLC. XOR, 0.2 - 1 mg, in 50 mM Bicine, pH 8.3, (0.5 ml), was placed in an anaerobic box and left to equilibrate for 1 h. To each sample, 250 mM anaerobic sodium dithionite (20 μl) and 500 mM anaerobic iodoacetamide (20 μl) were added and mixed before addition of the solution to anaerobic guanidinium hydrochloride to make a final concentration of 8M. The samples were wrapped in foil and incubated at room temperature for approximately 18 h in the anaerobic box. 40% (w/v) ammonium sulphate was added to each sample, the stoppered sample tubes were taken out of the anaerobic box and mixed at 4°C by rotation for 30 min before centrifugation (13000g, 30 min) at 4°C. The supernatant was applied to a Sep-Pak tC18 Cartridge (Waters) washed with H_2O (3 ml) and eluted in methanol (3 ml) at a flow rate of 1 ml/minute. The samples were evaporated to dryness in a rotary evaporator, redissolved in H_2O (500μl), and injected onto a vydac C18 10 μ reversed phase HPLC column and chromatographed isocratically with 0.1M ammonium acetate as the mobile phase at 1 ml/min in a Waters HPLC system. Detection was using a Waters multiwavelength detector at 280 and 367nm and peak heights were used to compare samples (Johnson *et al.*, 1990).

10.2.4 Inactivation of HMXOR by desulphuration

HMXOR in 50 mM Na Bicine, pH 8.3, was inactivated by incubation with KCN. To a solution of HMXOR, KCN was added to a final concentration of 10 mM (stock 100 mM KCN in 50 mM NaOH) and incubated at 37°C. Activity was followed by taking aliquots of the solution and assaying for xanthine oxidase activity (Section 3.2.3). When no activity was observed, usually after 2 h, the sample was gel filtered using a G25 PD10 column (Pharmacia).

10.2.5 Resulphuration of HMXOR

HMXOR, (0.5 mg) in 50 mM MOPS, pH 7.3, (1 ml) was placed in an anaerobic box, (<5 PPM O_2), and allowed to equilibrate for 1 h. After equilibration, anaerobic methyl viologen and sodium sulphide were added to final concentrations of 0.2 mM and 2 mM respectively. The solution was then titrated with aliquots of 1 mM sodium dithionite (5 μl) until a faint blue colour was observed upon reduction of the methyl viologen. The enzyme was then incubated at room temperature and regular aliquots were removed, gel filtered through a G25 PD10 column (Pharmacia), taken out of the anaerobic box and assayed for xanthine oxidase activity (Section 3.2.3)

10.3 Results

10.3.1 Colourimetric molybdenum estimations

The spectra of XOR samples were determined and the samples were appropriately diluted for the estimation of molybdenum. The spectra of the molybdenum dimercaptotoluene extracts were taken (Fig 10.1) and used in the calculation of molybdenum content.

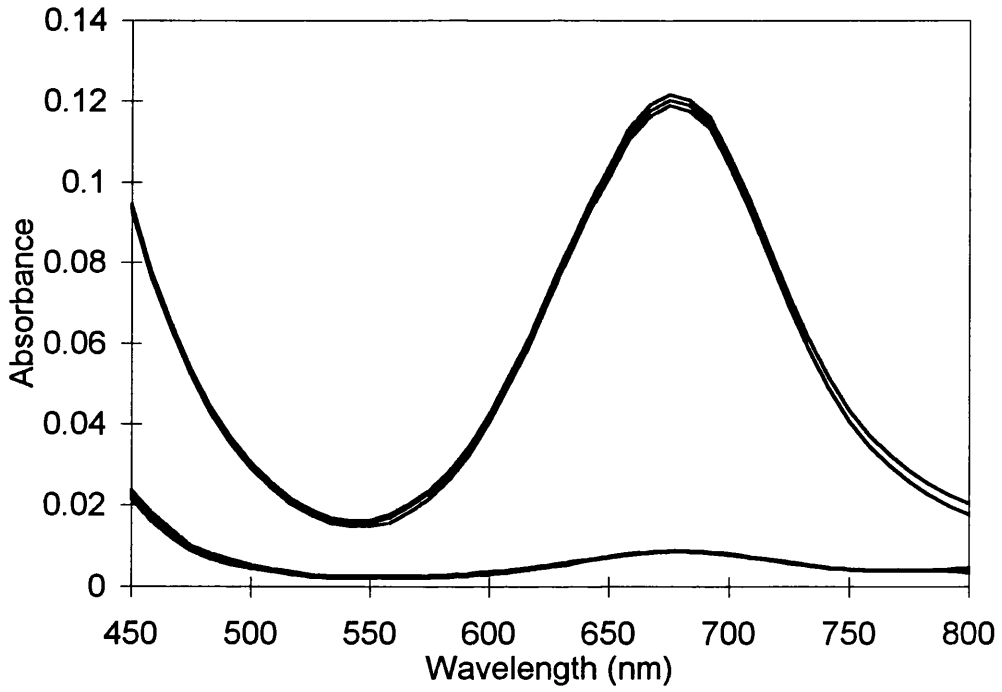


Fig. 10.1. Spectra of molybdenum dimercaptotoluene amyl acetate extracts. Spectra shown are triplicates of standards (10 μmoles Mo); upper spectra, and blanks; lower spectra.

The results are shown in Table 10.1, where molybdenum content is expressed per subunit, assuming each subunit contains 1 molecule of FAD.

Enzyme Sample and Preparation Number	Mo/XO (colourmetric)	Mo/XO (Mass Spec)
BMXOR 1	0.63 ± 0.04	0.61 ± 0.02
BMXOR 2	0.78 ± 0.02	
BMXOR 3	0.59 ± 0.02	
BMXOR 4 demolybdo	0.22 ± 0.01	
BMXOR 5 demolybdo	0.25 ± 0.01	
HMXOR 1	0.03 ± 0.00	0.06 ± 0.01
HMXOR 2	0.04 ± 0.01	
HMXOR 3	0.04 ± 0.01	
HMXOR 4	0.03 ± 0.01	
HMXOR 5	0.05 ± 0.04	
HMXOR 6	0.04 ± 0.01	
HMXOR 7	0.02 ± 0.02	
HMXOR 8	0.04 ± 0.01	

Table 10.1. Content of molybdenum in samples of HMXOR and BMXOR. Molybdenum contents were determined colourimetrically (Section 10.2.1) and by using mass spectroscopy (Section 10.2.2). Molybdenum contents are expressed as a fraction of XOR monomer.

The analyses show that the molybdenum content of HMXOR varied between 0.02 and 0.05, with a mean value for 8 samples of 0.038 ± 0.009 Mo/XO. BMXOR purified by myself from local milk gave a value of 0.59 ± 0.02 Mo/XO. Commercially-available BMXOR gave a similar value of 0.63 ± 0.04 Mo/XO and molybdenum-enriched BMXOR from folate affinity chromatography (Section 4.3.5) contained 0.78 ± 0.02 Mo/XO. In two separate preparations of demolybdo BMXOR using folate affinity chromatography chromatography contents of 0.22 ± 0.01 and 0.25 ± 0.01 Mo/XO were obtained.

10.3.2 Mass spectroscopy molybdenum estimations

The results, in Table 10.1, show that the estimations of molybdenum by mass spectroscopy gave very similar figures to those of the colourimetric analyses.

10.3.3 Molybdenum cofactor estimation

The inherent instability of the cofactor in air and light made exact quantification difficult and large losses in the cofactor were encountered. Between the two estimations, a 7 fold difference in yield was observed and the yield of di-cam Moco was relatively lower at higher concentrations of XOR. The Moco content of BMXOR in both analyses was assumed to

be 100% and the relative amount in HMXOR was calculated. Table 10.2 shows that the cofactor content of HMXOR was approximately 25% that of BMXOR, but because of large errors it is difficult to be fully confident of this value.

Analysis	Enzyme Sample	Peak height / mg	% BMXOR
1	BMXOR 1	0.0018 ± 0.0006	100 ± 30
	HMXOR 1	0.0005 ± 0.0002	27 ± 31
2	BMXOR 1	0.0132 ± 0.0031	100 ± 23
	HMXOR 1	0.0031	25

Table 10.2. Molybdenum cofactor content of HMXOR and BMXOR. The molybdenum cofactor was estimated by derivatisation and measurement of the 280nm peak height from HPLC (Section 10.2.3). The peak heights are expressed per mg XOR used per assay. To enable comparison between the two analyses, the peak heights / mg XOR are expressed as a percentage of BMXOR.

10.3.4 Estimations of inactive molybdenum-containing forms in HMXOR and BMXOR, based on xanthine oxidase activities and molybdenum contents.

The specific xanthine oxidase activities of HMXOR and BMXOR were normalised to 100% Mo content and, using a theoretical maximum AFR of 210 (Massey *et al.*, 1970), the percentages of inactive molybdenum-containing enzyme were calculated. Table 10.3 shows comparable specific activities for HMXOR and BMXOR, suggesting similarly high contents (50 - 60%) of desulpho enzyme.

	HMXOR 1	BMXOR 3
Xanthine oxidase activity (nmol/min/mg)	87.2 ± 4.4	1377.0 ± 23.5
Mo/FAD	0.04 ± 0.00	0.59 ± 0.02
Xanthine oxidase activity (nmol/min/mg 100% Mo)	2180 ± 110	2334 ± 40
% desulpho	59 ± 2	56 ± 1

Table. 10.3. Molybdenum-specific activities of HMXOR and BMXOR.
The total xanthine oxidase activity of samples of HMXOR and BMXOR were assayed and, using the known molybdenum contents, converted into specific activities per mg of molybdenum-containing enzyme. Assuming a fully functional AFR of 210, the percentage of inactive molybdenum was calculated.

10.3.5 Resulphuration of HMXOR

In view of the indications (Section 10.3.4) that HMXOR, in addition to containing a very high percentage of demolybdo enzyme, also has high contents of desulpho form, *in vitro* activation of the latter was attempted. HMXOR and HMXOR that had been previously-inactivated by cyanide treatment were separately resulphurated. Specific xanthine oxidase activities were determined (Section 3.2.3) before and after inactivation and after resulphuration (Table 10.4). Also the specific xanthine oxidase activity of HMXOR was monitored during the resulphuration process (Figure 10.2). Cyanide treatment of HMXOR caused total loss of activity after 2 h incubation. Both the active and cyanide-inactivated HMXOR samples regained more than their original activity, indicating that the original HMXOR sample contained desulpho enzyme (see Discussion)

Sample	Activity expressed as % of HMXOR
HMXOR	100% ± 0%
HMXOR after cyanide-inactivation	0% ± 0%
HMXOR after resulphuration	147% ± 3%
Cyanide-inactivated HMXOR after resulphuration	127% ± 2%

Table 10.4. The activities of cyanide-inactivated and resulphurated HMXOR. The values are expressed as a percentage of the activity of untreated HMXOR.

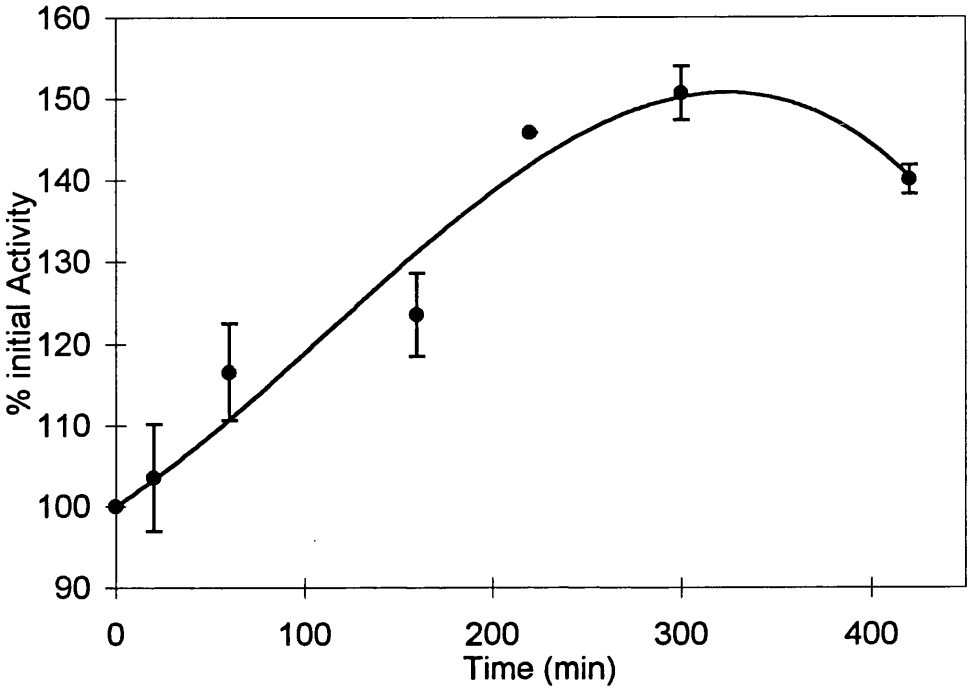


Fig. 10.2. Resulphuration of active HMXOR. HMXOR was resulphurated and the process was monitored by measuring xanthine oxidase activity throughout the timecourse. The activity is expressed as a percentage of that of the initial enzyme. Error bars relate to standard errors with n=3.

10.4 Discussion

HMXOR was shown to have a molybdenum deficiency of approximately 95%, BMXOR had a deficiency 40%; a not unusual value, as similar molybdenum deficiencies have been reported in many samples of BMXOR purified by a variety of methods (Hart *et al.*, 1970; Avis *et al.*, 1956 a). The low molybdenum content of HMXOR is however highly unusual, such values only having been previously reported in samples of fractionated demolybdo BMXOR (Gardlik *et al.*, 1987; Ventom *et al.*, 1988) or in XOR derived from specially tungsten-fed rats (Johnson *et al.*, 1974). The estimation of the molybdenum cofactor gave lower values for HMXOR than it did for BMXOR, indicating that, like demolybdo enzyme from other sources, HMXOR is also deficient in the molybdenum cofactor (Gardlik *et al.*, 1987; Ventom *et al.*, 1988). Unfortunately precise quantification was not possible due to the large errors in the assay.

Molybdenum deficiencies of HMXOR and BMXOR did not fully account for their specific xanthine oxidase activities, suggesting that a proportion of both enzymes contained molybdenum in the desulpho inactive form. This was confirmed, in the case of HMXOR, by the resulphuration of both active and cyanide-treated enzyme. Resulphuration of the cyanide-treated HMXOR achieved a specific activity of 127% that of the original enzyme while the specific activity of the untreated HMXOR increased to 147% of the original. Previously, it has been reported that upon the resulphuration

of cyanide-inactivated enzyme the specific activity will only increase to half of the theoretical activity maximum (Nishino *et al.*, 1982). Therefore a 27% increase would mean that the total theoretical maximum was 254% of the original. Therefore the calculated active enzyme content would be 39% (100%/254%) in the original sample. Similarly, the active enzyme content of the untreated enzyme is calculated to be 34% (100%/294%). The difference between the two figures could result partly from slight overall inactivation of the enzyme during the cyanide-inactivation process. These values for desulpho content are in reasonable agreement with those (Table 10.3) derived from enzyme activities and molybdenum contents.

11 Discussion

11.1 Purification of XOR

The preparation of XOR from bovine milk was found to yield approximately 24 mg XOR per l milk, comparable to amounts (19 mg/l), previously reported, (Hunt & Massey, 1992). Using an essentially identical preparation procedure, from fresh human milk, similar yields were obtained. However, the logistics of human milk collection dictated the use of frozen milk, from which yields of enzyme were initially only approximately 5 mg XOR per l milk, four fold lower than from fresh milk. The problem was addressed and improved conditions, involving addition of DTT, were developed that facilitated release of XOR from the membrane. The modified preparation yielded 21 mg HMXOR per l frozen milk; amounts comparable to those from fresh human or bovine milk.

The pterin oxidase activity in bovine milk was found to be approximately 27800 nmol min^{-1} l^{-1} whereas that in thawed human milk was 23 fold lower at approximately 1200 nmol min^{-1} l^{-1}. Purified BMXOR and HMXOR were found to have specific xanthine oxidase activities in the respective ranges of 1200 - 1500 nmol min^{-1} mg^{-1} and 30 - 110 nmol min^{-1} mg^{-1} which, using average values, represents a 20 fold lower specific activity of HMXOR compared with BMXOR. These values of specific activity were a little

higher than those (1060 nmol min^{-1} mg^{-1} and 24 nmol min^{-1} mg^{-1}) previously reported in our lab for BMXOR and HMXOR (Abadeh *et al.*, 1992), but nevertheless similar, in the case of BMXOR, to the value (1480 nmol min^{-1} mg^{-1}) reported by Hunt and Massey (1992). Sarnesto *et al.* (1996) reported, on the basis of ELISA and enzyme activity assay, that the XOR in human milk had a specific activity of 600 nmol min^{-1} mg^{-1}; a value considerably higher than that obtained in the present study for purified enzyme. However, in Sarnesto *et al.* assays were carried out at 37°C, whereas in this study they were done at 25 °C.

The above comparisons relate to the question as to whether the very low xanthine (or pterin) oxidase activity in purified HMXOR reflects loss of activity during the preparation procedure. The occurrence of such inactivation is not supported by the data. Thus, based on similar yields of purified enzyme from human and bovine milk, the ratios of HMXOR to BMXOR specific activities in whole milk and purified enzymes are comparable. Moreover, repeated freeze thawing was shown not to decrease the specific activity of HMXOR (data not shown). From this I concluded that the enzyme purified from human milk has not been significantly inactivated by freezing or during the preparation, and therefore that the low specific activity of purified HMXOR represents that *in vivo*. Concerning the data of Sarnesto *et al.* (1996), after adjustment for assay temperatures, their specific activity for XOR in fresh human milk is

approximately twice that obtained by me for purified enzyme. A difference that could well reflect different samples of breast milk.

The low specific activity of XOR in milk is not unique in human tissue. Similarly low specific activity XOR was identified in preliminary purifications from human heart (Abadeh *et al.*, 1993) and the specific activity shown by ELISA and activity assays in cultured human epithelial cells was 45 nmol min^{-1} mg^{-1} (Page *et al.*, 1998). Moreover, low activity toward conventional reducing substrates in other whole human tissues also indicates similarities with the human milk enzyme (Parks & Granger, 1986). Human liver and intestine appear to be exceptional in showing high catalytic rates toward conventional reducing substrates (Parks & Granger, 1986), suggesting that XOR in these tissues has relatively high specific activity. This idea is supported by the specific activity (1800 nmol min^{-1} mg^{-1}) of XOR purified from post-mortem human liver (Krenitsky *et al.*, 1986) and by values quoted for human liver (2700 nmol min^{-1} mg^{-1}) and intestine (3000 nmol min^{-1} mg^{-1}) by Sarnesto *et al.* (1996) on the basis of ELISA and activity assays of whole tissue homogenates.

11.2 Inactive forms of HMXOR

In this study, the molecular basis for the low specific activity of HMXOR was addressed. Abadeh *et al.* (1992) noted that, whereas the xanthine oxidase activities of HMXOR and BMXOR differed by 50 fold, their activities toward NADH as reducing substrate were similar; facts that they attributed to the presence of large amounts of molybdenum-inactive enzyme in their HMXOR preparation. Enzyme forms with an inactive molybdenum centre are indeed well known. Desulpho-XOR lacks a catalytically essential Mo=S group, replaced by Mo=O, while demolybdo-XOR lacks molybdenum and possibly also its cofactor. Both of these inactive forms have been shown to be present in preparations of enzyme from a variety of sources.

The present study re-evaluated the molecular basis for the low activity of HMXOR initially by estimating the molybdenum contents of different samples of HMXOR, one of which was a specially-prepared "big batch" on which several different assays could be performed. Molybdenum contents were determined both by using the colourimetric method of Hart *et al.* (1970) and by ICP-MS. The two methods produced comparable results and showed that, in HMXOR, greater than 95% of the molybdenum is absent, compared to an approximate deficiency of 37% for BMXOR.

The presence of such large amounts of demolybdo-enzyme was shown to account for most, but not all of the low activity of HMXOR. The content of the molybdenum-inactive desulpho-enzyme was therefore investigated. Resulphuration of HMXOR showed an increase in specific activity of 47%. On this basis, the desulpho-content of molybdenum-containing enzyme was estimated to be 61%, a value that fits well with the estimation of 59% from known molybdenum and activity data. These values are further supported by EPR data on my "big batch" HMXOR sample (Bray, personal communication).

The presence of the molybdenum cofactor (Moco), in HMXOR was also investigated as previous reports have indicated its absence in demolybdo-BMXOR (Gardlik *et al.*, 1987; Ventom *et al.*, 1988). Moco was found to be of the order of 75% lower in HMXOR than in BMXOR, However, because of large inaccuracies in the assay procedure I was unable to determine the precise content for either enzyme.

11.3 Spectral analyses

UV-Visible spectroscopy revealed significant differences between HMXOR and BMXOR, suggesting differences in their respective chromophores. Given that the former has a very much lower content of molybdenum, and possibly cofactor, it was initially attractive to attribute differences on this basis. However, quantification of the extinction coefficients, based on FAD estimations, allowed an accurate difference spectrum between HMXOR and BMXOR to be obtained. This difference spectrum was found to be remarkably similar to the spectrum of Fe-S protein II from *Azobacter vinelandii* (Shethna *et al.*, 1968) and of deflavo BMXOR (Komai *et al.*, 1969), suggesting that spectral differences between HMXOR and BMXOR could be largely attributable to a relative lack of Fe-S in HMXOR. Comparison of $\in_{450}$ values led to estimated deficiencies in Fe-S centre contents of 4 % and 30 % respectively for BMXOR and HMXOR, relative to molar equivalence with FAD. Confirmation of these estimated Fe-S deficiencies was obtained by computed addition of the spectra of FAD and BMXOR so as to reproduce the calculated Fe-S/FAD ratio of HMXOR, assuming negligible contribution to the spectra of molybdenum or Moco. By these means, the HMXOR visible spectrum was simulated with remarkable accuracy.

CD Spectroscopy was carried out on HMXOR, BMXOR and a sample of demolybdo-BMXOR, prepared by fractionation of BMXOR on a folate-affinity column. It is known that, in CD spectra of XOR, the main Cotton effects are largely attributable to Fe-S, with only small contributions from FAD, molybdenum and Moco (Bayer *et al.*, 1971; Komai *et al.*, 1969). From CD spectra, matched on a molar basis, it was seen that HMXOR and demolybdo-BMXOR samples have significantly smaller Cotton effects than does BMXOR. Normalisation of these spectra for Fe-S content, as determined above, led to spectra very similar in form and intensity, confirming calculations based on the UV-visible absorbance data.

CD spectra, both before and after Fe-S normalisation, showed relatively small differences below 350 nm. Previously, these have been attributed to differences in Moco content (Gardlik *et al.*, 1987); an explanation consistent with the findings of the present study. After normalisation for Fe-S, a HMXOR - BMXOR difference spectrum was obtained which showed negative and positive cotton effects at 500 nm and 575 nm respectively. This difference spectra was remarkably similar in form to that of Mo^{IV} - Mo^{VI} for BMXOR (Ryan *et al.*, 1995), suggesting that my difference spectrum relates to the CD spectrum of Mo^{VI} in XOR.

11.4 Fe-S deficiency

Gardlik *et al.* (1987), working with demolybdo-BMXOR, observed a lack of Fe-S, which, on the basis of EPR parameters, they attributed specifically to a lack of the Fe-S I centre and, to some extent, a replacement of this centre by a new Fe-S centre. Working with my HMXOR, Bray (personal communication) has arrived at similar conclusions. As a significant Fe-S deficiency has not been noted in any other system, this is of great interest. In the present studies, large deficiencies of Fe-S were observed not only with HMXOR, but also with demolybdo-BMXOR, suggesting a possible relationship between lack of Fe-S and molybdenum. The distance between the molybdenum and Fe-S I in BMXOR has been estimated, by magnetic coupling, to be 8 - 14 Å, considerably closer than any other two pairs of redox centres (Lowe & Bray, 1978). In our laboratory, efforts are underway to model the structure of HMXOR using co-ordinates derived for the related enzyme, aldehyde oxidoreductase (Romao *et al.*, 1995). Much of this structure has been modelled and, as shown in Fig 11.1 (Pearson, personal communication), Fe-S I and Moco are contiguous, with an Fe-S I-associated cysteine residue (Cys 139) in particular, being located very close to Moco.

Fig. 11.1. The molybdenum and iron sulphur I centres in HXO.

This intimate contact between Fe-S I and Moco adds credence to a link in deficiencies of these cofactors and to the possibility that the absence of Moco is able to alter the Fe-S I centre in such a way as to change its EPR parameters. The present treatment of the data may imply that Fe-S deficiency or alteration is a consequence of Moco deficiency. It is, however, conceivable, on the available evidence, that the reverse applies, in that changes in Fe-S lead to the absence of Moco.

11.5 Substrate specificities

Other than the low specific activity, largely attributed to the lack of molybdenum, the possibility remained that HMXOR was essentially identical to human liver XOR. Krenitsky *et al.* (1986) compared the wide substrate specificities of HLXOR and BMXOR and observed significant kinetic differences between the two enzymes. In the present work, these assays were repeated with HMXOR and BMXOR, using xanthine, hypoxanthine purine and 2-APR. Substrate specificity reflects substrate binding sites on XOR and can give information regarding differences between HMXOR and BMXOR, irrespective of different activity levels dependent on molybdenum content. In my hands the relative activities of BMXOR toward the above substrates were shown to be generally similar to those found by Krenitsky *et al.* (1986). HMXOR showed significant differences from BMXOR, and was found to be more similar to HLXOR based on the data of Krenitsky *et al.* (1986).

The primary structure of HMXOR is currently being determined in our laboratory and, although still lacking 200 of 1300 residues (Briggs & Pearson, unpublished data), it is very much closer to that of HLXOR (Ichida *et al.*, 1993) than to that of BMXOR (Berglund *et al.*, 1996) (see Appendix Section 12.3). The presently-described specificity data are clearly consistent with this.

11.6 Molecular weight of HMXOR

Abadeh *et al.* (1992), using gel filtration, reported the molecular weight of HMXOR to be slightly higher than that of BMXOR, and, in our laboratory, HMXOR has consistently shown a lower mobility on SDS-PAGE compared with BMXOR. The molecular weights of HMXOR and HLXOR have been quoted as approximately 300 kDa on the basis of gel filtration and native-PAGE (Krenitsky et al 1986; Sarnesto et al 1996). However in these studies human enzyme was not compared directly with that from other sources. In the present work, SDS-PAGE, gel filtration, dynamic light scattering (DLS) and native-PAGE all demonstrated a consistently higher molecular weight of HMXOR compared with BMXOR. However, the calculated molecular weights from the published sequences of BMXOR (Berglund *et al.*, 1996) and HLXOR (Ichida *et al.*, 1993), which is essentially identical to HMXOR (see Section 11.5), are very similar; a conclusion confirmed in the cases of HMXOR and BMXOR by MALDI-TOF MS data (Bray, personal communication). This indicates that the differences in apparent molecular weight reported here and previously result from factors other than mass. Gel filtration, DLS and native-PAGE are all susceptible to the tertiary structure of proteins, whereby a less spherical and compact enzyme appears to have a higher molecular weight. As a consequence of a lack of molybdenum and Moco, it is conceivable that HMXOR is partially unfolded and therefore appears,

when using the techniques described above, to be larger. However, as the bovine enzyme used in this study also contained significant quantities of demolybdo-enzyme, one may expect these techniques to reveal two, albeit close, components of different molecular weights. This was not observed. An apparent difference in mobility on SDS-PAGE cannot be explained in this way, as the proteins are completely denatured under these conditions. This technique is however susceptible to the primary structure of proteins. For example, mobility can be affected by a very large charge or proline-rich regions. The primary structures of BMXOR and HLXOR, which is essentially identical to the known sequence of HMXOR, contain very similar numbers of charged residues. However 9 out of 32 of the residues 193 - 225 of BMXOR are prolines, which correspond to the loop region between the Fe-S and FAD domains (Sato *et al.*, 1995). This region is similar in HLXOR, yet a full comparison of the amino acid sequence of HMXOR awaits data for the first 200 amino acids, and it will be interesting to compare the sequence of HMXOR in this region.

11.7 Aggregation of HMXOR

Differences in the stabilities of HLXOR and BMXOR after freezing and storage were noted by Krenitsky *et al.* (1986), and Abadeh *et al.* (1992) observed that HMXOR aggregated under these conditions. Demolybdo-BMXOR was found to be more susceptible to denaturation than active BMXOR in the presence of salicylate (Bergel & Bray, 1956). In the present work, it was shown that repeated freeze-thawing of HMXOR did not significantly affect its specific activity, although it was seen to increase the amount of aggregation, as judged by increased loss of protein after centrifugation (data not shown). This aggregation was much more pronounced than for BMXOR and not attributable to denaturation. The nature of this aggregation remains unclear. It is, like that of many other proteins, concentration dependent. It can be prevented and, once initiated, partially reversed by addition of low concentrations of NaCl, suggesting that the aggregation is electrostatic in nature. DTT was shown to dissolve the centrifugate from aggregated HMXOR and to disaggregate it in a time-dependant manner. The time course of this process was similar to that involved in the XO to XDH conversion, indicating that reduction of disulphides is somehow involved in disaggregation.

11.8 Heparin affinity

XOR has been shown to bind to heparin-agarose. This binding has been shown to be electrostatic in nature, and to involve lysine residues of XOR. Fukushima *et al.* (1995) isolated heparin-binding peptides, proteolytically-derived from HMXOR, identified them by amino acid sequencing and found them to contain lysine residues. My analysis of the data is that the equivalent peptides in aldehyde oxidoreductase are exposed on the exterior surface of the enzyme. Adachi *et al.* (1993) demonstrated that HMXOR will bind to endothelial cells in a manner analogous to its binding to heparin-agarose and proposed that this binding is via membrane-associated glycosaminoglycans. I have shown that DTT-reduced HMXOR has a lower affinity for heparin than does untreated enzyme. These differences do not simply reflect the presence of oxidase or dehydrogenase forms, because DTT-treatment can lower the affinity of proteolysed oxidase enzyme to that of the non-proteolysed dehydrogenase form. It is worthy of note that circulating XOR is most likely to be in the oxidase form and so best suited to bind to endothelial cell membranes, which may then be injured by XOR-generated ROS (Koiij *et al.*, 1994).

In many respects differences in heparin affinity between BMXOR and HMXOR follow the same pattern as aggregation. I have shown a considerable species difference in the tendencies of XOR to aggregate,

HMXOR has a considerably higher affinity for heparin than does BMXOR. In fact, at pH 7.4, BMXOR will not bind to heparin-agarose beads (Radi *et al.*, 1997). Aggregation of HMXOR can be reduced by addition of DTT, as can heparin affinity, and both aggregation and heparin binding can be reduced by NaCl. Clearly, both aggregation and heparin-binding properties are dependant on conformation, itself subject to modification by sulphydryl oxidation. It is conceivable that the increased aggregation and heparin-binding we observe of HMXOR compared with BMXOR results of an increased exposure of a positively-charged region. Such a region might not only bind to sulphated glycosaminoglycans, but could also facilitate aggregation by binding to its negatively-charged neighbours.

11.9 Oxidase and dehydrogenase interconversions

The oxidase form of XOR is generally considered to be the source of destructive ROS whether in the inflammatory response or in pathological states involving I-R (Kooij *et al.*, 1994). While XDH to XO interconversions have been well studied for BMXOR and rat liver XOR, very little has been reported concerning human XOR. Sanders *et al.* (1997) demonstrated that proteolysis of HMXOR can produce very largely oxidase form enzyme; an observation confirmed it the present study. However, there is no published information concerning non-proteolysed HMXO, although the interconversions of human XOR are generally assumed to be identical to those of the well-studied rat liver and bovine milk enzymes. I compared the non-proteolytic interconversions of HMXOR and BMXOR and found this not to be the case.

The DTT-induced oxidase to dehydrogenase conversions of HMXO and BMXO were found to be very similar to each other The reverse conversions of BMXDH to BMXO were generally as described in previous reports (Nakamura & Yamakazi 1982; Hunt & Massey, 1992) and similar to those of the rat liver enzyme (Della Corte & Stirpe, 1971; Waud & Rajagopalan 1976 b; Saito, 1987), HMXOR, however was different in that HMXDH to HMXO conversion was not only slower but much less complete.

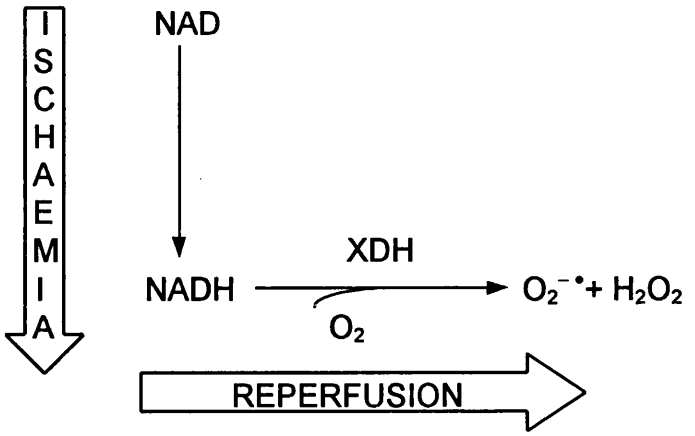
Waud and Rajagopalan (1976 b) described the conversion for the enzyme from rat liver with DTDP, which showed that the rapid oxidation of 14 sulphydryl groups was complete after 20 minutes, whereas the conversion was only complete after 270 minutes. These workers concluded that there were two phases in the conversion, the oxidation of sulphydryls followed by a slower conformational change. Like the rat liver enzyme, DTT-reduced HMXOR was found to contain 14 free sulphydryl groups per subunit which are rapidly oxidised by DTDP, yet even after 24h incubation the oxidase activity only accounted for 64% of the total, compared with a value of 98% for BMXOR. The number of free cysteines in a HMXO preparation rich in oxidase form (75%) was estimated to be 7.6 ± 1.0 per subunit, which gave a difference between fully reduced enzyme of 6 sulphydryl groups per subunit, similar to the number (7 per subunit) quoted for the dehydrogenase to oxidase conversion of BMXOR and rat liver XOR (Saito, 1987; Hunt & Massey, 1992). The finding that the same number of sulphydryl groups is available for oxidation in HMXOR as in enzymes from other sources largely exclude this as a factor in the incomplete conversion to HMXO (Saito, 1987). Moreover, the fact that the high oxidase enzyme could be obtained through proteolysis indicate that, unlike the avian enzyme (Sato et al., 1995), formation of highly oxidase form was possible. A remaining explanation is that the conformational change required after oxidation of sulphydryl groups is less favoured than that for bovine milk and rat liver enzymes.

11.10 Significance of inactive XOR

The large proportions of demolybdo-XOR in human milk and, by analogy, in tissues other than liver and intestine, suggests a physiological significance. It is known that purine catabolism is strictly regulated as purine biosynthesis is very energy consuming and the majority of purines are salvaged. Less active XOR will certainly help in this respect. Inactive enzyme may, however, be activated if necessary. Inactive XOR in the liver of chickens can be activated as the protein content of the diet is increased. Itoh *et al.*, (1978) attributed this activation to desulpho-sulpho conversion (see Section 1.4.2); a process for which enzyme-catalysed mechanisms have been proposed (Nishino *et al.*, 1983). Page *et al.* (1988) showed that the enzyme in a human mammary epithelial cell line has low xanthine oxidase activity, similar to that found in human milk. However, upon administration of the inflammatory cytokine interferon γ, an 800% increase in activity was observed, which was shown to result partly from an approximately 3-fold post translational activation. The specific activity in unstimulated cells was shown by ELISA and activity assays to be 45 nmol min^{-1} mg^{-1} and therefore would increase to 144 nmol min^{-1} mg^{-1}. Even after this increase, the vast majority of the protein must still be assumed to be inactive, when comparison is made with BMXOR with a specific activity of 1500 nmol min^{-1} mg^{-1}. Also relevant to these discussions are the findings of Brown *et al*, (1995) who showed that

xanthine oxidase activity in human milk peaked during the first 15 days after birth and fell thereafter; an increase attributable to an increase in specific activity, as corresponding changes in XOR protein were not observed. The extent of activation observed (maximum rate of 85 nmol min^{-1} mg^{-1}) again places the specific activity increase within the scope of the low molybdenum content in my samples of HMXOR and allows a desulpho-sulpho conversion to account for their activation.

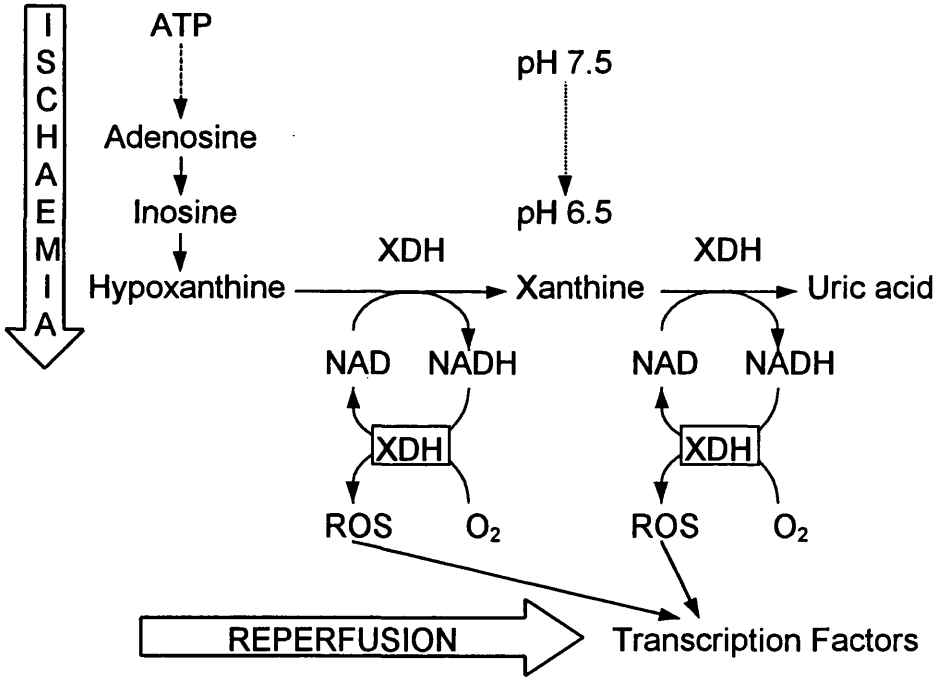
One ROS-producing activity that is not affected greatly by the form of the enzyme or by lack of molybdenum is the NADH oxidase activity, which , considering the large amounts of inactive enzyme present in some tissues, may have a physiological role. The concentration of cytoplasmic NADH is 1μM, whereas NAD^+ is 400-500 μM (Tischler *et al.*, 1977). During ischemia there is an increase in cytoplasmic levels of NADH, with the NAD/NADH ratio decreasing from 700 to 30 (Nishino & Tamura, 1991). If the same substrate pool is available after ischemia, then the cytoplasmic NADH concentration could increase to 13-17 μM. On this basis, it has been postulated (Harrison, 1997; Zhang *et al.*, 1998) that NADH oxidase activity, triggered by increased NADH concentrations in ischemia, may replace or complement the much discussed xanthine oxidase hypothesis (Scheme 11.1).



Scheme 11.1. The mechanism for production of ROS from NADH by XOR during ischemia. Modified from Harrison (1997).

My observation that a low molybdenum-active enzyme in the dehydrogenase form can very efficiently produce ROS from xanthine oxidation, in the presence of low concentrations of NAD^+ may also have some relevance here. I-R injury has been shown to be greatly reduced by administration of specific XOR molybdenum centre inhibitors. During ischemia, NADH levels rise, as do the levels of hypoxanthine and xanthine, which rapidly decrease during reperfusion (Xia & Zweier, 1995). Also during ischemia, the pH of tissues falls to approximately 6.5 (Herman *et al.*, 1990), a pH which favours the oxidation of NADH (Fig. 8.1) and the proposed recycling back to NAD^+. On this basis I propose an alternative hypothesis for I-R injury and physiological ROS production in tissues containing XOR of low specific activity which accounts for all the above observations, does not depend upon a dehydrogenase to oxidase

conversion and is consistent with blockage of ROS production and I-R Injury by molybdenum centre inhibitors. During Ischemia, as described by Granger *et al.* (1981), there is a net catabolism of ATP, producing hypoxanthine and xanthine, and as the pH drops these are oxidised by XOR producing NADH, which accumulates. Upon reperfusion demolybdo-XOR rapidly oxidises NADH, which is replaced at a similar rate by the oxidation of xanthine and hypoxanthine. ROS generated from NADH oxidation upregulate transcription factors, increase ICAM production and neutrophil adhesion and so promote consequential damage (Scheme 11.2).



Scheme 11.2. Proposed new mechanism for production of ROS from largely demolybdo enzyme.

A similar scheme is also relevant to cells under normal physiological conditions as I have demonstrated that the same recycling mechanism can operate, albeit less efficiently, at higher pH. Because all the reducing equivalents derive from xanthine this mechanism allows inactivation and activation of the molybdenum centre via a sulpho-desulpho switch to control the overall rate of ROS production.

Although not explored in the present study, this scheme may also be relevant to higher activity enzymes, such as those observed in bovine milk and rat liver, which are still 50 - 70% inactive. In situations of non-saturating substrate concentrations, for example at 0.5 μM xanthine, or a considerably higher concentration of a substrate with a correspondingly lower V_{max}, the xanthine:NADH turnover of the inactive enzyme would be sufficiently low as to be comparable to the rate of NADH:NAD^+ turnover of the inactive enzyme.

This demonstration of the production of ROS from the dehydrogenase form of XOR, omits the necessity of the increasingly questionable dehydrogenase to oxidase conversions that many of the proposed hypotheses of XOR in physiological and pathological conditions depend upon, and provides a relevance for the large amounts of molybdenum-inactive XOR found.

12 Appendix

12.1 Sequence alignment of HLXOR and BMXOR.

HLXOR (Ichida *et al.*, 1993); top sequence

BMXOR (Berglund *et al.*, 1996); bottom sequence

```
MTADKLVFFV NGRKVVEKNA DPETTLLAYL RRKLGLSGTK LGCGEGGCGA CTVMLSKYDR
********** ** ******* ********** ****** *** ********** **********
MTADELVFFV NGKKVVEKNA DPETTLLAYL RRKLGLRGTK LGCGEGGCGA CTVMLSKYDR

LQNKIVHFSA NACLAPICSL HHVAVTTVEG IGSTKTRLHP VQERIAKSHG SQCGFCTPGI
***** **** ******** * ********** ********** ********** **********
LQDKIIHFSA NACLAPICTL HHVAVTTVEG IGSTKTRLHP VQERIAKSHG SQCGFCTPGI

VMSMYTLLRN QPEPTMEEIE NAFQGNLCRC TGYRPILQGF RTFARDGGCC GGDGNNPNCC
********** ***** ****  ********* ********** ****  **** ** *******
VMSMYTLLRN QPEPTVEEIE DAFQGNLCRC TGYRPILQGF RTFAKNGGCC GGNGNNPNCC

MNQKKDHSVS HSPSLFKPEE FTPLDPTQEP IFPPELLRLK DTPRKQLRFE RERVTWIQAS
******* *   ***** *** * ******** ********** * * ******  *********
MNQKKDHTVT LSPSLFNPEE FMPLDPTQEP IFPPELLRLK DVPPKQLRFE GERVTWIQAS

TLKELLDLKA QHPDAKLVVG NTEIGIEMKF KNMLFPMIVC PAWIPELNSV EHGPDGISFG
********** *** ****** ********** ** ***** * ******** * **** *****
TLKELLDLKA QHPEAKLVVG NTEIGIEMKF KNQLFPMIIC PAWIPELNAV EHGPEGISFG

AACPLSIVEK TLVDAVAKLP AQKTEVFRGV LEQLRWFAGK QVKSVASVGG NIITASPISD
*** ** *** **  ******  ********* ********** ******* ** **********
AACALSSVEK TLLEAVAKLP TQKTEVFRGV LEQLRWFAGK QVKSVASLGG NIITASPISD

LNPVFMASGA KLTLVSRGTR RTVQMDHTFF PGYRKTLLSP EEILLSIEIP YSREGEYFSA
*********  *** ****** *** ****** * ****** * ********** **** * ***
LNPVFMASGT KLTIVSRGTR RTVPMDHTFF PSYRKTLLGP EEILLSIEIP YSREDEFFSA

FKQASRREDD IAKVTSGMRV LFKPGTTEVQ ELALCYGGMA NRTISALKTT QRQLSKLWKE
********** ***** **** ** **   *  **********  ********* * ****** *
FKQASRREDD IAKVTCGMRV LFQPGSMQVK ELALCYGGMA DRTISALKTT QKQLSKFWNE
```

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ELLQDVCAGL AEELHLPPDA PGGMVDFRCT LTLSFFFKFY LTVLQKLGQE NLEDKCGKLD
 ********** **** * *** ****  ** * ********** **** ***      *******
KLLQDVCAGL AEELSLSPDA PGGMIEFRRT LTLSFFFKFY LTVLKKLGKD SK DKCGKLD

PTFASATLLF QKDPPADVQL FQEVPKGQSE EDMVGRPLPH LAADMQASGE AVYCDDIPRY
**  ****** ** ***  ** *********  ** ******* *** ****** **********
PTYTSATLLF QKHPPANIQL FQEVPNGQSK EDTVGRPLPH LAAAMQASGE AVYCDDIPRY

ENELSLRLVT STRAHAKIKS IDTSEAKKVP GFVCFISADD VPGSNITGIC NDETVFAKDK
**** ***** ********** ** *** *** ***** ****  **** **   *********
ENELFLRLVT STRAHAKIKS IDVSEAQKVP GFVCFLSADD IPGSNETGLF NDETVFAKDT

VTCVGHIIGA VVADTPEHTQ RAAQGVKITY EELPAIITIE DAIKNNSFYG PELKIEKGDL
********** ********   ***  ** ** * ******** **********  *********
VTCVGHIIGA VVADTPEHAE RAAHVVKVTY EDLPAIITIE DAIKNNSFYG SELKIEKGDL

KKGFSEADNV VSGEIYIGGQ EHFYLETHCT IAVPKGEAGE MELFVSTQNT MKTQSFVAKM
********** **** *****  ********* ** **** ** *********  **********
KKGFSEADNV VSGELYIGGQ DHFYLETHCT IAIPKGEEGE MELFVSTQNA MKTQSFVAKM

LGVPANRIVV RVKRMGGGFG GKETRSTVVS TAVALAAYKT GRPVRCMLDR DEDMLITGGR
**** *** * ********** ******* **  ********* **********  *********
LGVPVNRILV RVKRMGGGFG GKETRSTLVS VAVALAAYKT GHPVRCMLDR NEDMLITGGR

HPFLARYKVG FMKTGTVVAL EVDHFSNVGN TQDLSQSIME RALFHMDNCY KIPNIRGTGR
********** ****** *** **** ** **   *** **** ********** **********
HPFLARYKVG FMKTGTIVAL EVDHYSNAGN SRDLSHSIME RALFHMDNCY KIPNIRGTGR

LCKTNLPSNT AFRGFGGPQG MLIAECWMSE VAVTCGMPAE EVRRKNLYKE GDLTHFNQKL
****** *** *********    *** **** ****** *** *** ** *** ******** *
LCKTNLSSNT AFRGFGGPQA LFIAENWMSE VAVTCGLPAE EVRWKNMYKE GDLTHFNQRL

EGFTLPRCWE ECLASSQYHA RKSEVDKFNK ENCWKKRGLC IIPTKFGISF TVPFLNQAGA
***  ****  *** **** * ********** ********** ********** **********
EGFSVPRCWD ECLKSSQYYA RKSEVDKFNK ENCWKKRGLC IIPTKFGISF TVPFLNQAGA

LLHVYTDGSV LLTHGGTEMG QGLHTKMVQV ASRALKIPTS KIYISETSTN TVPNTSPTAA
* ******** *  ******* ********** ** ***** * ********** **** *****
LIHVYTDGSV LVSHGGTEMG QGLHTKMVQV ASKALKIPIS KIYISETSTN TVPNSSPTAA

SVSADLNGQA VYAACQTILK RLEPYKKKNP SGSWEDWVTA AYMDTVSLSA TGFYRTPNLG
*** *  *** *** ****** **** *****  ******* * ** * ****  **********
SVSTDIYGQA VYEACQTILK RLEPFKKKNP DGSWEDWVMA AYQDRVSLST TGFYRTPNLG

YSFETNSGNR FHYFSYGVAC SEVEIDCLTG DHKNLRTDIV MDVGSSLNPA IDIGQVEGAF
*********  **** ***** ********** ********** ********** **********
YSFETNSGNA FHYFTYGVAC SEVEIDCLTG DHKNLRTDIV MDVGSSLNPA IDIGQVEGAF
```

```
VQGLGLFTLE ELHYSPEGSL HTRGPSTYKI PAFGSIPIEF RVSLLRDCPN KKAIYASKAV
********** ********** ********** ******* ** ********** **********
VQGLGLFTLE ELHYSPEGSL HTRGPSTYKI PAFGSIPTEF RVSLLRDCPN KKAIYASKAV


GEPPLFLAAS IFFAIKDAIR AARAQHTGNN VKELFRLDSP ATPEKIRNAC VDKFTTLCVT
******* **  ********* ******* **  ********* ********** **********
GEPPLFLGAS VFFAIKDAIR AARAQHTNNN TKELFRLDSP ATPEKIRNAC VDKFTTLCVT


GVPENCKPWS VRV
* * ******  **
GAPGNCKPWS LRV
```

12.2 Sequence alignment of AOR and HLXOR.

AOR (Thoenes *et al.*, 1994); top sequence

HLXOR (Ichida *et al.*, 1993); bottom sequence

```
..MIQKVITV NG...IEQNL FVDAEALLSD VLRQQLGLTG VKVGCEQGQC GACSVI....
      *  * **    * *    * *  *    **  *** *  * **  * * *** *
MTADKLVFFV NGRKVVEKN. .ADPETTLLA YLRRKLGLSG TKLGCGEGGC GACTVMLSKY

..LDGKVVR.........ACVT KMKRVADGAQ ITTIEGVGQP EN.LHPLQKA WVLHGGAQCG
  *  * *          *        **      ** ** *      *** *        * ***
DRLQNKIVHF SANACLAPIC SLHHVA.... VTTVEGIGST KTRLHPVQER IAKSHGSQCG

FCSPGFIVSA KGLLDTNADP SREDVRDWFQ KHRNACRCTG YKPLVDAVM............
** **   *    **     *   *     **    * ***** * *
FCTPGIVMSM YTLLRNQPEP TMEEIENAFQ G..NLCRCTG YRPILQGFRT FARDGGCCGG

...DAAAVIN GKK..... .......... .......... .......... ..........
          *  **
DGNNPNCCMN QKKDHSVSHS PSLFKPEEFT PLDPTQEPIF PPELLRLKDT PRKQLRFERE

.......... .......... .........P ETDLEFKM.. .......... ..........
                                  *   * *
RVTWIQASTL KELLDLKAQH PDAKLVVGNT EIGIEMKFKN MLFPMIVCPA WIPELNSVEH

PADGRIWGSK YP........ .......... .......... .......... ..........
  **   *    *
GPDGISFGAA CPLSIVEKTL VDAVAKLPAQ KTEVFRGVLE QLRWFAGKQV KSVASVGGNI

.......... .......... .......... .......... .......... ..........

ITASPISDLN PVFMASGAKL TLVSRGTRRT VQMDHTFFPG YRKTLLSPEE ILLSIEIPYS

.......... ....RPTAVA KVTGTL.... .......... .......... ..........
               *    * ***
REGEYFSAFK QASRREDDIA KVTSGMRVLF KPGTTEVQEL ALCYGGMANR TISALKTTQR

.......... ..DYGADLG. .LKMPAGT.. .......... .......... ..........
            *  * *    *  *
QLSKLWKEEL LQDVCAGLAE ELHLPPDAPG GMVDFRCTLT LSFFFKFYLT VLQKLGQENL

.......... .......... .......... .......... .......... ..........

EDKCGKLDPT FASATLLFQK DPPADVQLFQ EVPKGQSEED MVGRPLPHLA ADMQASGEAV
```

```
.......... .LHLAMVQAK VSHANIKGID TSEALTMPGV HSVITHKDVK GKNRITGLIT
           * *  *      ** ** ** ****   **     *   **  * * ***
YCDDIPRYEN ELSLRLVTST RAHAKIKSID TSEAKKVPGF VCFISADDVP GSN.ITGICN

FPTNKGDGWD RPILCDEKVF QYGDCIALVC ADSEANARAA AEKVKVDLEE LPAYMSGPAA
         *        **    *  *  *  **       * *  **   ** **
.........D ETVFAKDKVT CVGHIIGAVV ADTPEHTQRA AQGVKITYEE LPAII.....

AAEDAIEIHP GTPNVYFEQP I.VKGEDTGP IFASADVTVE GDFYVGRQPH MPIEPDVAFA
  ****       *                  *  **  *  *  * *  *    *     *
TIEDAIK... ..NNSFYGPE LKIEKGDLKK GFSEADNVVS GEIYIGGQEH FYLETHCTIA

Y.MGDDGKCY IHSKSIGVHL HLYMIAPGVG LEPDQLVLVA NPMGGTFGYK ..FSPTSEAL
   *  *                        *       *      *** ** *    *
VPKGEAGEME LFVSTQNTMK TQSFVAKMLG VPANRIVVRV KRMGGGFGGK ETRSTVVSTA

VAVAAMATGR PVHLRYNYQQ QQQYTGKRSP WEMNVKFAAK KDGTLLAMES DWLVDHGPYS
** **  *** **              ** * *      *     * **  * *  *     *
VALAAYKTGR PVRCMLDRDE DMLITGGRHP FLARYKVGFM KTGTVVALEV DHFSNVGNTQ

EFGDLLTLRG AQFIGAGYNI PNIRGLGRTV ATNHVWGSAF RGYGAPQSMF ASECLMDMLA
                 * * ***** **    **     ** ** * ** *    ** *   *
DLSQSIMERA LFHMDNCYKI PNIRGTGRLC KTNLPSNTAF RGFGGPQGML IAECWMSEVA

EKLGMDPLEL RYKNAYRPGD TNPTGQEPEV FSLPDMIDQL RPKYQ..... AALEKAQKES
   **   *  * ** *  **       *  *  * **           *          *  **
VTCGMPAEEV RRKNLYKEGD LTHFNQKLEG FTLPRCWEEC LASSQYHARK SEVDKFNKEN

TATHKKGVGI SIGVYGSAWT ........AL TPPQPGPSSM PTAPSPCIRP GKTMARARTS
    * *  *      *   *          **        *    *          *
.CWKKRGLCI IPTKFGISFT VPFLNQAGAL LHVYTDGSVL LTHGGTEMGQ GLHTKMVQV.

AAWARRTKPC VPWAWLRKRS SSPGPTPPPP PTPAPPAWAE QVMTGNAIRV ACENLLKACE
*  *              *    *   *   *       *        * *    **   **  *
ASRALKIPTS .......KIY ISETSTNTVP NTSPTAASVS ADLNGQAVYA ACQTILKRLE

.....KPGGG YYTYDELKAA DKPTKITGNW TASGATHCDA VTGLGKPFVV YMYGVFMAEV
     ** *             *                      *  *  *     ***   **
PYKKKKPSGS WEDWVTAAYM DTVSLSATGF YRTPNLGYSF ETNSGNRFHY FSYGVACSEV

TVTVATGRPP WTALTLMADL GSLCNQLATD GQIYGGLAQG IGLALSEDFE DIKKHATLVG
     **           *  **  *       **  *    *  **   *          *
EIDCLTGDHK NLRTDIVMDV GSSLNPAIDI GQVEGAFVQG LGLFTLEEL. HYSPEGSLHT

AG.....FPF IKQIPDKLDI VYV.NHPRPD GPFGASGVGE LPL...TSPH AAIINAIKSA
 *      *     **                        ***  **    *    **  **  *
RGPSTYKIPA FGSIPIEFRV SLLRDCPNKK AIYASKAVGE PPLFLAASIF FAIKDAIRAA

.......TGV RIYRL..PAY PEKVLEYLKA
              **  **  ***
RAQHTGNNVK ELFRLDSPAT PEKIRNACVD KFTTLCVTGV PENCKPWSVR V
```

12.3 Sequence alignment of HMXOR and HLXOR.

HMXOR (Briggs & Pearson, unpublished data); top sequence

HLXOR (Ichida *et al.*, 1993); bottom sequence

```
MTADKLVFFV NGRKVVEKNA DPETTLLAYL RRKLGLSGTK LGCGEGGCGA CTVMLSKYDR

LQNKIVHFSA NACLAPICSL HHVAVTTVEG IGSTKTRLHP VQERIAKSHG SQCGFCTPGI

VMSMYTLLRN QPEPTMEEIE NAFQGNLCRC TGYRPILQGF RTFARDGGCC GGDGNNPNCC

                       TPLDPTQEP IFPPELLRLK DTPRKQLRFE GERVTWIQAS
                       ********* ********** **********  *********
MNQKKDHSVS HSPSLFKPEE FTPLDPTQEP IFPPELLRLK DTPRKQLRFE RERVTWIQAS

TLKELLDLKA QHPDAKLVVG NTEIGIEMKF KNMLFPMIVC PAWIPELNSV EHGPDGISFG
********** ********** ********** ********** ********** **********
TLKELLDLKA QHPDAKLVVG NTEIGIEMKF KNMLFPMIVC PAWIPELNSV EHGPDGISFG

AACPLSIVEK TLVDAVAKLP AQKTEVFRGV LEQLRWFAGK QVKSVASVGG NIITASPISD
********** ********** ********** ********** ********** **********
AACPLSIVEK TLVDAVAKLP AQKTEVFRGV LEQLRWFAGK QVKSVASVGG NIITASPISD

LNPVFMASGA KLTLVSRGTR RTVQMDHTFF PGYRKTLLSP EEILLSIEIP YSREGEYFSA
********** ********** ********** ********** ********** **********
LNPVFMASGA KLTLVSRGTR RTVQMDHTFF PGYRKTLLSP EEILLSIEIP YSREGEYFSA

FKQASRREDD IAKVTSGMRV LFKPGTTEVQ ELALCYGGMA NRTISALKTT QRQLSKLWKE
********** ********** ********** ********** ********** **********
FKQASRREDD IAKVTSGMRV LFKPGTTEVQ ELALCYGGMA NRTISALKTT QRQLSKLWKE

ELLQDVCAGL AEELHLPPDA PGGMVDFRCT LTLSFFFKFY LXVXXKLGQE NLEDKCGKLD
********** ********** ********** ********** * *  ***** **********
ELLQDVCAGL AEELHLPPDA PGGMVDFRCT LTLSFFFKFY LTVLQKLGQE NLEDKCGKLD

PTFASATLLF QKDPPADVQL FQEVPKGQSE EDMVGRPLPH LAADMQASGE AVYCDDIPRY
********** ********** ********** ********** ********** **********
PTFASATLLF QKDPPADVQL FQEVPKGQSE EDMVGRPLPH LAADMQASGE AVYCDDIPRY

ENELSLRLVT STRAHAKIKS IDTSEAKKVP GFVCFISADD VPGSNITGIC NDETVFAKDK
********** ********** ********** ********** ********** **********
ENELSLRLVT STRAHAKIKS IDTSEAKKVP GFVCFISADD VPGSNITGIC NDETVFAKDK
```

```
VTCVGHIIGA VVADTPEHTQ RAAQGVKITY EELPAIITIE DAIKNNSFYG PELKIEKGDL
********** ********** ********** ********** ********** **********
VTCVGHIIGA VVADTPEHTQ RAAQGVKITY EELPAIITIE DAIKNNSFYG PELKIEKGDL

KKGFSEADNV VSGEIYIGGQ EHFYLETHCT IAVPKGEAGE MELFVSTQNT MKTQSFVAKM
********** ********** ********** ********** ********** **********
KKGFSEADNV VSGEIYIGGQ EHFYLETHCT IAVPKGEAGE MELFVSTQNT MKTQSFVAKM

LGVPANRIVV RVKRMGGGFG GKETRSTVVS TAVALAAYKT GRPVRCMLDR DEDMLITGGR
********** ********** ********** ********** ********** **********
LGVPANRIVV RVKRMGGGFG GKETRSTVVS TAVALAAYKT GRPVRCMLDR DEDMLITGGR

HPFLARYKVG FMKTGTVVAL EVDHFSNVGN TQDLSQSIME RALFHMDNCY KIPNIRGTGR
********** ********** ********** ********** ********** **********
HPFLARYKVG FMKTGTVVAL EVDHFSNVGN TQDLSQSIME RALFHMDNCY KIPNIRGTGR

LCKTNLPSNT AFRGFGGPQG MLIAECWMSE VAVTCGMPAE EVRRKNLYKE GDLTHFNQKL
********** ********** ********** ********** ********** **********
LCKTNLPSNT AFRGFGGPQG MLIAECWMSE VAVTCGMPAE EVRRKNLYKE GDLTHFNQKL

EGFTLPRCWE ECLASSQYHA RKSEVDKFNK ENCWKKRGLC IIPTKFGISF TVPFLNQAGA
********** ********** ********** ********** ********** **********
EGFTLPRCWE ECLASSQYHA RKSEVDKFNK ENCWKKRGLC IIPTKFGISF TVPFLNQAGA

LLHVYTDGSV LLTHGGTEMG QGLHTKMVQV ASRALKIPTS KIYISETSTN TVPNTSPTAA
********** ********** ********** ********** ********** **********
LLHVYTDGSV LLTHGGTEMG QGLHTKMVQV ASRALKIPTS KIYISETSTN TVPNTSPTAA

SVSADLNGQA VYAACQTILK RLEPYKKKNP SGSWEDWVTA AYMDTVSLSA TGFYRTPNLG
********** ********** ********** ********** ********** **********
SVSADLNGQA VYAACQTILK RLEPYKKKNP SGSWEDWVTA AYMDTVSLSA TGFYRTPNLG

YSFETNSGXP FHYFSYGVAC SEVEIDCLTG DHKNLRTDIV MDVGSSLNPA IDIGQVEGAF
********   ********** ********** ********** ********** **********
YSFETNSGNR FHYFSYGVAC SEVEIDCLTG DHKNLRTDIV MDVGSSLNPA IDIGQVEGAF

VQGLGLFTLE ELHYSPEGSL HTRGPSTYKI PAFGSIPIEF RVSLLRDCPN KKAIYASKAV
********** ********** ********** ********** ********** **********
VQGLGLFTLE ELHYSPEGSL HTRGPSTYKI PAFGSIPIEF RVSLLRDCPN KKAIYASKAV

GEPPLFLAAS IFFAIKDAIR AARAQHTGNN VKELFRLDSP ATPEKIRNAC VDKFTTLCVT
********** ********** ********** ********** ********** **********
GEPPLFLAAS IFFAIKDAIR AARAQHTGNN VKELFRLDSP ATPEKIRNAC VDKFTTLCVT

GVPENXKPXL XXR
***** **
GVPENCKPWS VRV
```

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